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AUTHOR ..... JAMES NEILSON

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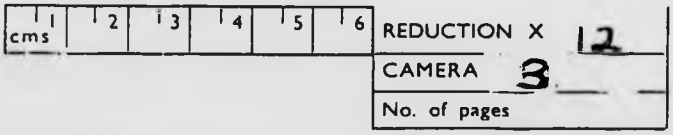

ATTACHMENT OF BACTERIA TO GLASS SURFACES IN PURE CULTURE AND IN MIXED SUSPENSIONS AND THE EFFECT OF GROWTH CONDITIONS ON THAT ATTACHMENT.

JAMES NEILSON

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

UNIVERSITY OF WARWICK DEPARTMENT OF BIOLOGICAL SCIENCES.

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CONTENTS

APPENDICES PAGE

APPENDIX TABLES

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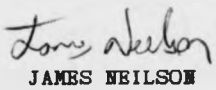
DECLARATION

I declare that this thesis is a report of the research undertaken by myself in the Department of Biological Sciences at Warwick University, under the supervision of Dr M Fletcher and Dr K Flint. It is my own work and includes nothing which is the outcome of work done in collaboration.



JAMES NEILSON

SUMMARY

The attachment of selected freshwater bacteria, *Aeromonas*, *Chromobacterium*, a coryneform and *Staphylococcus* in pure culture and in mixed suspensions with one other bacterium to glass surfaces was investigated in the laboratory.

Changes in the nutrient conditions of the growth medium during growth and attachment and the temperature, pH and electrolyte type and concentration present in the attachment solution during attachment experiments all influenced bacterial attachment. The pure culture attachment results obtained depended on the bacterial species being investigated. When bacterial species were attached in the presence of one other species the growth conditions still had a profound affect on attachment. The results obtained depended on the bacterial species present in the mixed suspension, with some bacterial species having a more profound affect on the attachment of other species than others.

The bacterial species used to study bacterial attachment in the laboratory were used along with *Acinetobacter* and *Pseudomonas* to investigate bacterial attachment *in vivo* using a model system. The attachment of these bacteria were investigated in two- to six-membered suspensions. The biofilms obtained in the model system consisted of smaller bacterial cells in a more densely packed biofilm. These biofilms could still be influenced by growth conditions as temperature was seen to influence the bacterial biofilm obtained. During these attachment experiments the *Acinetobacter* which did not attach itself had a profound affect on the attachment of other bacterial species when present in the liquid phase.

The mechanism by which *Acinetobacter* inhibited the attachment of other bacteria was investigated and it appeared *Acinetobacter* did not excreate a chemical to influence the attachment of other species, but the *Acinetobacter* cells themselves had to be present in the liquid phase to influence the attachment of other species.

The effects of different molecular weight fractions of Tocil Lake water were investigated for their affect on bacterial attachment. The fraction containing the > 30,000 MW component was found to influence bacterial attachment. The results obtained, depended on the bacterial species being investigated.

CHAPTER ONE

INTRODUCTION

1.1 BIOFILM IMPORTANCE

Bacterial attachment is an important and widespread phenomenon and research has shown that only a few surfaces in the natural environment cannot be colonised by bacteria. These are rare and are always surfaces of plants or animals, e.g. Sieburth (1975), showed that the body of a hydra was free of attached bacteria but the stalk was colonised by numerous bacteria. The apical tips of the brown algae Ascophyllum nodosum have also been shown to be free of attached bacteria whereas old growth was colonised by bacteria (Cundell, 1977). Variations in bacterial attachment to these living surfaces were thought to be due to modifications of the living tissue being used as a surface as it ages.

Bacterial biofilms present on a surface can have detrimental consequences for humans. Sphaerotilus natans has been found growing on surfaces in water delivery systems such as the surfaces of industrial heat exchanges (McCoy et al. 1982). These biofilms have been shown to cause energy loss due to heat transfer, accelerated corrosion and clogging in the heat exchange systems (Pedersen, 1982). During oil recovery in the petroleum industry, water is sometimes injected into reservoirs to enhance oil recovery. The bacteria present in this water have been known to cause plugging of pipelines used in this process (Cerini et al. 1946; Clementz et al. 1982). This is due to fluid frictional resistances, a result of which is that the cross sectional area available for flow is reduced due to the attached bacteria and the surface roughness and drag forces of the surface are increased (Picologlou et al. 1980). Attachment is also important when

investigating how plant pathogens cause disease in their fruit hosts. These diseases result in the fruits being unsuitable for human consumption and this will be important when considering agricultural economics (Jones, 1990).

There are numerous examples of bacterial diseases resulting from the adherence of microorganisms to the surfaces of cells, disorders such as cholera, gonorrhoea and endocarditis, all need specific attachment of the pathogen to the host tissues (Lankford, 1960; Ward et al. 1972; Gould et al. 1975). One of the most common examples of bacterial attachment in humans is the interactions of oral steptococci and the surfaces of teeth. These bacteria initiate dental diseases (caries) by the demineralisation of the teeth due to acid production in a localised area (Hsieh et al. 1985; Doyle et al. 1982; Kolenbrander, 1990). Other medical problems can arise due to bacterial adherence to prosthetic devices such as cardiac valves, pacemakers, catheters and joint prostheses. The colonisation of these devices by bacteria, such as Staphylococcus species, will usually lead to an infection (Costerton, 1978; Jacques et al. 1987).

The attachment phenonemon can also be a problem during microbiological experiments. A good example of this is the attachment of an adhesive mutant to the surface of the vessel in an artifical culture system like a chemostat which then becomes the dominant bacterial population by ousting the parental organism from the system. Further bacteria originating in this culture will be derived mainly from wall growth by sloughing or propagation into the medium (Larsen et al. 1964).

Bacterial biofilms, however, can also be advantageous to humans. Examples of bacterial biofilms that have been used to man's advantage

include those in trickling filters which are used to treat wastewater, leading to the removal of enteric bacteria and viruses and also to the decomposition of organic matter, allowing the discharge of relatively clean sewage effluent into the river system (Mack etal, 1975). Water from water treatment plants must also be monitored. Stewart (1990) has shown that bacteria attached to activated carbon are very resistant to disinfection and these bacteria could cause problems if they were to get into effluent from the water treatment plant. Attached bacteria can be exploited for their metabolic capabilities, a good example is the exploitation of bacterial methanogenic activity for methane production (Harvey et al. 1984). Bacterial enzymes can also be more efficient in industrial applications if the bacterium is attached. An example of this is in the production of chemical feedstocks through the enzymatic hydrolysis of plant biomass and other waste materials (Duff et al. 1985).

As the field of bacterial biofilm development is so large this introduction will look mainly at biofilm development on solid surfaces in freshwater and marine environments. Research has shown that microbial colonisation of a solid surface in a marine or a freshwater environment follows a distinct succession. This suggests that as the biofilm develops there is a change in the abundance and the species composition of the bacteria present in the biofilm.

Copiotrophic bacteria which cannot grow in low nutrient conditions but can react quickly to high nutrient levels, (Poindexter, 1981), are the first group of bacteria found in a developing biofilm (Marshall et al, 1971b; Corpe, 1973). Good examples of these primary colonisers are Pseudomonas species and Flavobacterium species. These bacteria are found

surrounded by a polymeric matrix in a biofilm, and this polymeric material can represent in some cases a large component of the biofilm (Corpe, 1973; Dempsey, 1981). Bacterial colonisation will continue with the appearance of oligotrophic bacteria which can grow in low nutrient environments, examples of these types of bacteria are, Hyphomicrobium species and Caulobacter species (Corpe, 1973). Photosynthetic algae and diatoms as well as larval forms of macro-organisms embedded in the polymeric matrix have been reported at this early stage (Crisp et al. 1960).

As the succession continues, the biomass, numbers and diversity of the attached microorganisms increase (Jordan et al. 1976). The quantity of polymeric matrix also increases during biofilm development, (Bryers et al. 1981), therefore the composition of the biofilm becomes more complex with time. In the latter stages of biofilm development and of the succession, microalgae followed by fungi, diatoms and protozoa are seen to appear (Jordan et al. 1976; Marszalek et al. 1979). Therefore in these later stages of biofilm development, there are primary producers present as well as the heterotrophic consumers and this could allow for the possible interaction between the different biofilm components.

These studies demonstrate how a developing biofilm can become a complex community in aquatic environments. This thesis is concerned with investigating one component of this biofilm, the attachment of bacteria to solid surfaces in freshwater aquatic environments. As bacteria are the initial colonisers of solid surfaces when introduced into aquatic environments, then these studies are concerned with the initial stages of biofilm development. Before the bacterial attachment process is

discussed in detail, the reasons why bacteria attach to solid surfaces in aquatic environments will be considered.

1.2 WHY BACTERIA ATTACH TO SURFACES

Research has demonstrated that attached bacteria can be more competitive in aquatic environments than free-living bacteria (Fletcher, 1979). However attachment may not always be benefical to a bacterium, as seen when bacteria attached to suspended particles in some aquatic environments are lost from the aquatic environment as these particles settle out of the liquid phase of the aquatic environment (Lovell, 1985). To aid in the understanding of why bacteria attach, a comparison of attached and free-living bacteria was made.

1.2.1 Comparison Between Attached and Free-Living Bacteria

In the open ocean, free-living bacteria predominate (Ferguson, 1976; Wibe et al. 1972), whilst in fresh and estuarine waters the numbers of attached bacteria have been shown to vary greatly (Bell et al. 1982; Geesey, 1979). The contribution made by attached bacteria and free-living bacteria to the total microbial process in the aquatic ecosystems is therefore difficult to evaluate due to the variations in the numbers of attached and free-living bacteria present in the system. Attached bacteria in estuarine environments can be seen to be active for longer periods of time compared to free-living bacteria. These attached bacteria also remain in estuarine environments longer than free-living bacteria. This could be due to the attached bacteria being trapped within the turbidity zone of the estuary, where suspended particles are concentrated due to a complex series of events within the estuary, while free-living bacteria can be dispersed by natural forces within the estuary. This could result in the free-living bacteria being flushed

from the estuarine environment (Clarke, 1980). Therefore free-living bacteria which play a role in the biological activity of the estuary would be lost from the estuarine environment as they are washed into the sea.. The fate of the bacteria lost from the estuary would depend on how good these bacteria were at adapting to their new environment. It is possible that these free-living bacteria would be more competitive in the new environment than attached bacteria. This could also occur when large numbers of bacteria are attached to particles. These particles could settle out and so another fraction of the aquatic bacterial population could be lost to the sediment. This will be of most significance in water systems with high particle content or if large amounts of particles are introduced into an aquatic system (Lovell, 1985).

Bacteria which are attached in aquatic environments are said to be more active than free-living bacteria (Fletcher, 1979; Harvey, 1980). Jeffrey (1986) demonstrated that the activity of attached bacteria is greater than that of free-living bacteria in oligotrophic environments, while glucose and glutamate incorporation by attached bacteria was greater than that observed for free-living bacteria in different coastal environments (Kirchman, 1982). Glucose assimilation by attached cells and the respiration of this glucose was also found to be greater for attached cells than free-living cells (Fletcher, 1986). These experiments also indicated that glucose assimilation by detached cells was greater than the assimilation by attached cells. This could be due to the cells having a bigger surface area for glucose assimilation when they are detached. Attached cells were activated or changed by the attachment process which enabled glucose to be assimilated more

actively. It is possible that the glucose transport system was modified by the attachment process (Fletcher, 1986; Iriberri, 1990). However, these differences between attached and detached cells have not been reported by other researchers (Bright, 1983), so their relevance is questionable.

Attached cells are thought to be less sensitive to change in nutrient conditions. If nutrients suddenly become plentiful in an aquatic system attached bacteria are seen to be less active than free-living bacteria. This could be due to the attached cells having less surface area for nutrient uptake than free-living bacteria (Jeffrey, 1986). Attached cells tend not to decrease in size like free-living bacteria responding to nutrient starvation. If marine bacteria are starved, they decrease their size, so that the surface area to volume ratio increases enormously (up to 50% higher than for comparable non-starved cells). Attached cells do not do this, because nutrient accumulation at surfaces tends not to lead to starvation conditions. Therefore, on detachment these cells have a larger surface area but a lower surface area-to-volume ratio. It is this ratio that is most important in determining uptake rates by bacteria. This greater activity by unattached bacteria has also been reported by Hattori et al (1960 + 1961). Higher succinate oxidation rates were observed for unattached Escherichia coli and Azotobacter agile compared to the values obtained for the attached species. The presence of inert particles in a liquid medium has been shown to increase bacterial activity (Jannasch, 1977) or decrease bacterial activity (Gordon, 1983). Therefore, the influence of surfaces on bacterial activity is unclear. It is likely that external factors will influence the response of a bacterium as much as the

presence of a surface, with the net result that attachment can be beneficial or detrimental to the bacterium.

Bell (1982) demonstrated that salinity, heterotrophic uptake and particle load were the main factors influencing differences between attached bacteria and free-living bacteria in different aquatic environments. Substrate and substrate concentration can also account for differences in the activity between attached bacteria and free-living bacteria (Bright, 1983; Meinhard, 1985). Seasonal variations within water bodies and the resulting environmental changes will influence the activity of attached and free-living bacteria (Meinhard, 1985). These results suggest that at different times of the year and in the presence of different substrates it is possible to get different parts of the bacterial community becoming active. Therefore the nutritional and environmental conditions present in a water body could select for the active bacterial population, and could possibly select for the bacterial populations attaching to solid surfaces introduced into this aquatic environment. This thesis will look into this proposal in greater detail and investigate if changing the nutritional and environmental conditions during bacterial attachment will influence the bacterial species attaching to solid surfaces in aquatic environments.

There are many reports of the shape and size of a bacterium varying when it becomes attached compared with its shape and size in the liquid phase (Fletcher, 1982; Meinhard, 1985). Bacteria have been shown to be larger in size when attached compared to free-living cells, which could account for any increase in activity. However, bacterial cells can also be smaller in size when attached compared to the liquid phase and this is thought to be related to the starvation response. Once the nutrients

present on a solid surface have been utilized by the attached bacteria starvation conditions would then be present. (Kjelleberg, 1983).

1.2.2 Survival and Attachment

The adhesion of bacteria to surfaces has often been described as a survival tactic. Dawson (1981) has shown that the number of cells of a Vibrio species attaching to a surface, increased with exposure to starvation. The presence of inert beads in a medium with a low nutrient concentration allowed attached bacteria to grow whereas bacteria in the liquid phase could not grow (Jannasch, 1972). Even the absence of a specific substrate can influence adhesion, a carbon-limited medium can increase attachment (Brown, 1977). Therefore bacterial attachment may be involved in overcoming starvation especially when a specific nutrient is involved. Stevenson (1978) said that attachment was an important "fitness trait" for aquatic bacteria. The ability of bacteria to attach to a surface could provide them with a new microenvironment which could be higher in nutrients than the surrounding environment, enabling them to survive starvation conditions. The ability of a bacterium to scavenge the nutrients associated with a surface is related to its ability to attach to the surface, which in turn will be influenced by other nutritional and environmental factors (Kjelleberg, 1983). As indicated in many bacterial attachment experiments, external factors will influence the attachment, and the same factors will affect the starvation process of bacteria.

The actual starvation process is important when studying attachment. Two stages can be observed in the starvation response in aquatic environments. The initial process is dwarfing which is marked by a decrease in cell volume which is the result of reductive division

(Novitsky, 1977) or utilisation of cellular products (Koch, 1971). This initial process is followed by a more stabilised starvation stage which is seen during long-term exposure to low nutrient conditions (Kjelleberg, 1983; Beverley, 1983). These dwarf cells both on the surface and in liquid media are capable of surviving long periods of time without nutrients (Novitsky, 1977). During starvation, the nutrients present on a surface will be used up very quickly. Therefore the surfaces will become nutrient limited. Studies have shown that the mucopeptide in the cell diminishes and the outer membrane of the bacterium changes during attachment under starvation conditions (Beverley, 1983). These changes in the outer components of the cell could be due to the attached bacteria utilising some of their own surface components. Perhaps attached bacteria can so this more efficiently than free-living bacteria, and therefore can survive starvation conditions better than free-living bacteria (Malmcroma-Friberg, 1986). Alternatively the bacteria could change their outer cell components to aid in the hunt for nutrients. Wrangstadh (1988) has demonstrated that a Pseudomonas species attached to a solid surface produced a polymer on the onset of starvation, which resulted in the Pseudomonas detaching from the solid surface. This polymer was thought to be part of a mechanism by which a bacterium could be detached from a surface and then go onto scavenge other sites on the surface for nutrients.

One of the main cell surface characteristics which can influence attachment and survival is the hydrophobicity of the cell surface. Kjelleberg (1983) has shown that the binding of dwarf cells was affected by hydrophobicity. Hydrophilic bacteria become smaller at a surface when

nutrients were limited compared to hydrophobic bacteria (Humphrey, 1983). Hydrophobic bacteria can still utilise nutrients at a surface. This could be due to changes in the cell surface during starvation which could then enable the hydrophobic bacteria to utilise the surface bound nutrients (Kefford, 1982). The importance of cell hydrophobicity on attachment will be discussed in detail later in this chapter.

These studies demonstrate that it is not always benefical for bacteria to attach to solid surfaces in aquatic environments. However, it is clear that attachment has a role to play when considering bacterial populations in aquatic environments.

1.3 ATTACHMENT PROCESS

There are three components of the aquatic attachment process which should be considered, the solid surface, the attached bacteria and the liquid environment. This thesis in part investigates how changing environmental and nutritional conditions in the liquid environment influence the attachment of bacteria to a surface. To aid in the understanding of the results obtained from these investigations it is important to have a good knowledge of the solid surface and bacteria involved in these attachment studies.

1.3.1 The Solid Surface

The characteristics of the solid surface involved in attachment experiments are important as they will influence the attachment process. The characteristics of a solid surface to be considered include the physico-chemical or physical characteristics of the solid surface.

1.3.1.(A) Physico-chemical Characteristics

A physicochemical characteristic of a solid surface which could influence bacterial attachment is the surface free energy of the solid

surface. Surface free energy is important as it includes all surface forces which could interact with forces in the other components of the attachment process e.g. the liquid environment or the bacteria. When considering this thermodynamic approach to the attachment process, free energy is the 'available' energy within a system. When considering a surface, free energy is a result of the molecules, groups or atoms which are unable to interact with other such molecules, groups or atoms which approach the surface (Fletcher, 1982). The significance of surface free energy in bacterial attachment can only been seen when the free energy within the system is considered;

$$\Delta F^{adh} = \gamma^{bs} - \gamma^{ls} - \gamma^{bl}$$

Where ΔF^{adh} is the change in free energy of adhesion and where γ^{bs}, γ^{ls} and γ^{bl} are the bacterium/solid, liquid/solid and bacterium/liquid interfacial energies respectively (Neumann, 1979; Fletcher, 1982). The interfacial energies in each case are determined by the surface free energies of the two phases. Generally, the larger the difference in surface energies between two phases, the larger the interfacial energies. When considering the system as a whole adhesion is favoured by a reduction in free energy e.g. a negative value for ΔF^{adh}. Gerson (1980) demonstrated that this thermodynamic model for bacterial attachment did work as the attachment of Serratia marcescens and two Staphylococcus species increased when the ΔF^{adh} value was negative compared to when the ΔF^{adh} value was positive.

In the laboratory the ΔF^{adh} value is hard to obtain, however, Neumann (1979) calculated ΔF^{adh} as a function of the surface tension of the surface (γ_{sv}). Therefore free energy (γ_s) of a surface could be approximated in the laboratory by determining the critical surface

tension. This can be done by using the equation-of-state approach outlined by Neumann (1974). Problems, experimental and theoretical, arise when evaluating the surface tensions of liquids, solids and the bacterial surface. Theoretically liquid should not interact with the solid surface, however, the molecules of the liquid and the bacterial cell can interact e.g. hydrogen bonding. Other problems can arise when measuring the surface tension of bacterial cells in the laboratory as decribed by Pethica (1980). These problems include cellular components being transferred to the liquid which alters the liquid surface tension. When the attachment process is considered as a whole other factors will affect attachment such as entropy (the degree of disorder within the system) and free energy changes in the bacterial surface must be considered (Fletcher, 1982). Therefore the use of surface tension to obtain the ΔF^{adh} can be questionable.

When two surfaces come together during the attachment process, the interaction between the free energy of the surfaces is not the only factor to be considered. When two surfaces come into contact, the interactive groups on the two surfaces must be complementary. Therefore a surface with few of polar groups would be unlikely to be attached to by a bacterial surface with little polar groups. Thermodynamic models applied to cellular adhesion also assume that electrical charges in the system can be ignored. Research has shown that changing the ionic strengh of a medium can change the levels of bacterial attachment (Gingell, 1978). These results indicated that electrostatic interactions could be involved in the attachment process.

Electrostatic attraction or repulsion can occur when the charged groups of the bacterial surface and those of the solid surface come into

contact. When the groups on each surface bear opposite charge, binding of the surfaces can occur. However, if similar charges are present then repulsion can occur. In Chapter 5, the primary and secondary minima are described when a bacterial surface and a solid surface come into contact. As bacteria and solid surfaces usually bear a net negative charge, the long range forces between them will tend to be repulsive. Adsorption can still occur with two like surfaces when the long range repulsion forces are balanced by van der Waal's attraction forces. Two distances of separation between the surfaces become apparant at which there is a net attraction, the primary and secondary minima (Chapter 5 and FIGURE 5.2). These two areas of attraction are separated by an area where repulsive forces will be hard for the bacteria to overcome.

The primary and secondary minima are thought to be important in bacterial attachment to a solid surface and will be discussed in greater detail in Chapter 5. However, in relation to the interactions being discussed here, it is thought that at the secondary minimum, bacteria are held a distance from the surface and that long range forces may be the interactions involved in interacting with the solid surface. When the primary minimum is considered however, short range forces are thought to be more important in interactions between the bacterial surface and the solid surface (Marshall, 1971; Absolom, 1983; Fletcher, 1985). These short range interactions include;

(a) van der Waals dispersion interactions, weak interactions caused by fluctuations in the spatial concentration and distribution of electrons in molecules or atoms.

(b) electrostatic interactions between charged groups.

(c) polar interactions between groups with permanent or induced

dipoles.

(d) chemical bonding e.g. ionic, covalent and hydrogen bonding.

(e) hydrophobic bonding.

When considering bacterial attachment to a solid surface, the bacterium must overcome the repulsion barrier and allow short range forces to play a part in the attachment process. This might not be possible with some bacteria, however, some bacterial species will have external appendages which will enable them to overcome these repulsion barriers and make contact with the solid surface. These appendages include pili and flagella which will be discussed in this chapter.

Of the short range forces investigated in the laboratory, hydrophobic bonding has been shown to be important in bacterial attachment to a solid surface. Hydrophobic bonding in aqueous solutions involves the interaction of non-polar groups of opposing surfaces. This is thought to occur due to the exculsion of non-polar groups from water due to the strong attraction between molecules of water rather than the attraction forces between the non-polar groups themselves (Tanford, 1979). Research has shown that marine and freshwater bacteria prefer to attach to hydrophobic (low energy) surfaces than hydrophilic (high energy) surfaces (Fletcher, 1979; Pringle, 1983). These results suggest that hydrophobic bonding could be involved in bacterial attachment to solid surfaces.

Fletcher (1983) demonstrated that a range of bacteria from freshwater attached least to the most hydrophobic and hydrophilic surfaces. These results question the importance of hydrophobic bonding in bacterial attachment to solid surfaces. As the bacterial surface can enter into numerous interactions with the solid surface as indicated in

this chapter, it is unlikely that only hydrophobic interactions will be involved in bacterial attachment. In these experiments (Fletcher, 1983), the attachment of bacteria to different solid surfaces varied with the bacterial species being investigated. When considering the bacterial attachment process as a whole it is not surprising that these interactions will be influenced by differences in bacterial surface characteristics. Other solid surface factors will also influence the attachment of bacteria. These include changes in the solid surface due to physical changes such as surface roughness or due to surface conditioning when introduced to the liquid environment.

1.3.1.(B) Physical Characteristics

Physico-chemical characteristics have been shown to influence the attachment of bacteria to solid surfaces. Therefore different surfaces with different physico-chemical characteristics will have varying numbers of attached bacteria. The attachment of bacteria to solid surfaces will also be influenced by physical factors such as surface roughness (Weise, 1978), which is discussed in Chapter 3. Another factor which must be considered when solid surfaces are immersed in liquid environment is the spontaneous adsorption of molecules at the solid surface interface which results in the formation of a surface conditioning film (Baier, 1981). This conditioning film could provide an area where nutrients are concentrated at the surface and are accessible to bacteria (Characklis, 1983). These nutrient could be used up very quickly by bacteria so this form of conditioning film could be only of short term importance with regard to the attachment process. These molecules could be made less accessible to bacteria if they have the opposite charge to the surface. This will result in these nutrients

being held at the surface by strong interaction forces. Faison (1990) has demonstrated surface energy to be important in determining the amount and the availability to bacteria of a protein at a surface. These nutrients may not be utilised by bacteria if they do not fit in with the nutritional characteristics of the bacteria concerned (Fletcher, 1984). Therefore these molecules could remain on the solid surface and influence the attachment of bacteria to these surfaces by other means such as changing the solid surface characteristics in some way.

When macromolecules are present in the conditioning film, they are usually irreversibly bound to the solid surface. These macromolecules will be less accessible to bacteria to be used as nutrients as bacterial enzymes will be required to break these macromolecules down before they could be utilised by bacteria. These macromolecules include proteins, glycoproteins, proteoglycans and polysaccharides. These molecules are often found in low concentrations in natural environments (Fletcher, 1982). Therefore the macromolecules present in these conditioning films could originate from other sources such as bacterial or other organisms excretory products or from the breakdown of dead cells. These macromolecules when present in the conditioning films have been shown to change the physico-chemical characteristics of a solid surface (Baier, 1981), therefore they will influence the attachment of bacteria to solid surfaces.

Norde (1978) suggested that the formation of a conditioning film will depend on the molecules and solid surfaces being investigated. Therefore the affinity of a given protein for a negatively charged surface will be influenced by protein characteristics such as;

(a) Hydrophobicity of the protein.

(b) Structural rearrangements of the protein.

(c) The number of positively charged groups located at the surface.

The affinity of the solid surface for the protein will also be important. This affinity would increase with;

(a) Increasing hydrophobicity of the surface.

(b) Lowering of the surface charge.

(c) Screening of the solid surface charge by specifically adsorbed cations.

The result of a conditioning film forming at a surface is that the surface will assume the net charge and characteristics of the outermost portion of the protein. These changes in the characteristics of the solid surface will lead to an increase or decrease in bacterial attachment to the solid surface.

Research has shown conditioning films do not always promote bacterial attachment to solid surfaces (Marshall, 1985). Fletcher (1984) demonstrated that various proteins inhibited the attachment of bacteria to solid surfaces when present on the solid surface. There is, however, alot of evidence to suggest that conditioning films aid or encourage bacteria to attach to solid surface. Bacteria have been shown to utilize compounds adsorbed onto solid surfaces (Kefford, 1982; Gordon, 1985) and the presence of these compounds on a solid surface could lead to an increase in attached bacteria on the surface. It has also been suggested that conditioning films may attract bacteria to surface, due to a bacterial chemotactic response (Harris, 1973). In these cases bacteria are not using the conditioning film compounds as nutrients, but the chemical compound themselves are enhancing bacterial attachment by some unknown mechanism.

1.3.2 The Bacterium and Attachment

The latter discussion has demonstrated that the solid surface itself can influence the attachment of bacteria to that solid surface. Research has also shown that different bacterial species under the same nutritional and environmental conditions vary in their ability to attach to the same solid surface (McEldowney, 1986). These results suggest that these bacterial species attach differently to the solid surface due to differences in the bacteria themselves.

Bacterial parameters which can influence bacterial attachment to solid surfaces are discussed throughout this thesis, however, when a bacterium comes into close proximity to a solid surface the bacterial surface would be expected to be important in the attachment process.

1.3.2.(A) The Bacterial Surface

The surface characteristics of a bacterial surface could be important in determining whether or not a particular bacterium attaches to a solid surface (Section 1.3.I.(A)). The surface components of the bacterial surface itself will determine these characteristics. The outer most layer of the outer membrane of a Gram-negative bacterium is the lipopolysaccharide layer (FIGURE 1.1). The outer most part of this is the oligosaccharide-containing variable region, which one could speculate would be the most important portion for attachment. Attached to the outer oligosaccharide core is an 'O' antigen polysaccharide side chain, and a glucosamine-containing lipid (Schlegel, 1986). Costerton (1974), has shown that parts of this complex are exculsively located on the outer most portion of the bacterial membrane and are therefore probably involved in attachment. Gram-negative bacteria, even in the same species, can produce varying amounts of lipopolysaccharide and

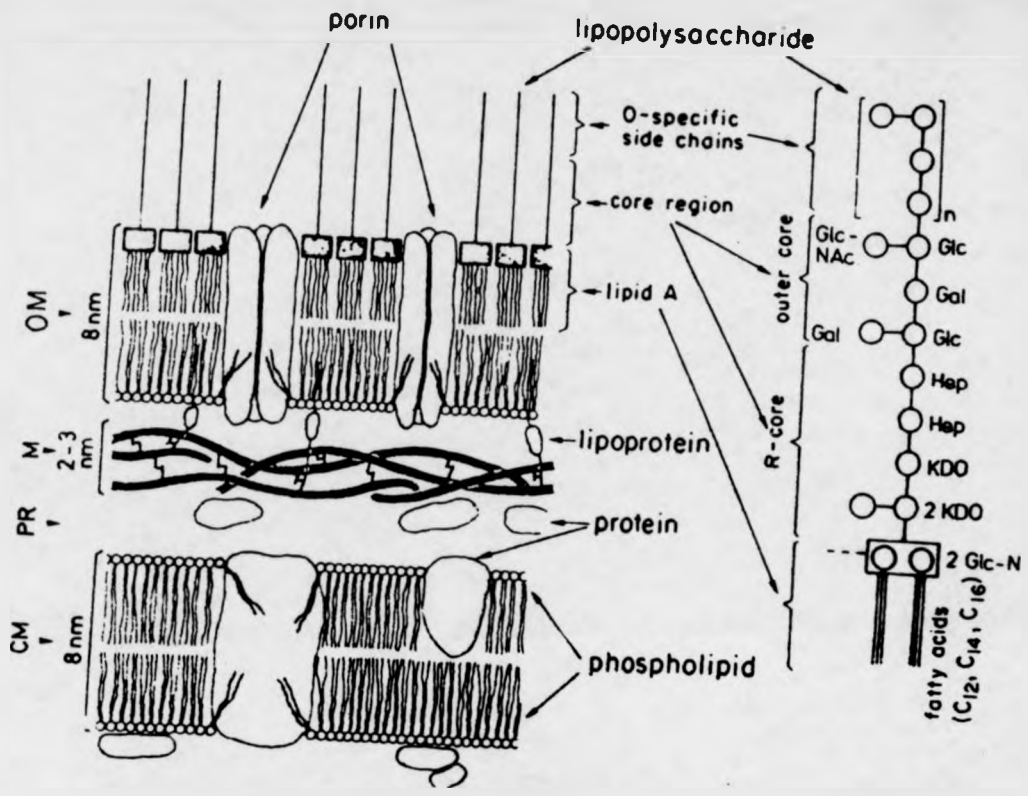


FIGURE 1.1
A model of the Gram-negative cell envelope or outer membrane (OM).
The cytoplasmic membrane (CM) is surrounded by the murein layer (M). The periplasmic space is the area separating the CM and M. Lipoproteins are embedded in a lipid layer which contains phospholipids and the lipid A zone of lipopolysaccharides.

A detailed structure of the lipopolysaccharide is shown on the right.
Glc - glucose,
Glc-N - glucosamine,
Glc-NAc - N-acetyl glucosamine,
Gal - galactose,
Hep - heptose,
KDO - 2-keto-3-deoxyoctanoic acid.

therefore their potential to attach to a solid surface could also vary (Shands, 1966).

The outer most layers of Gram-positive bacteria are different from those of the Gram-negative bacteria. In Gram-negative bacteria, the murein is present as a single layer and represents less than 10% of the cell wall dry weight. In Gram-positive bacteria, murein represents 30-70% of the cell dry weight and consists of about 40 layers. In Gram-positive bacteria, the tetrapeptide side chains of the muramic acid are connected by interpeptide chains and the amino acids present in these chains vary with the bacteria species being investigated. These different amino acids could transfer different surface characteristics to the different bacterial species. Teichoic acid is also present in Gram-positive outer layers, this is not found in Gram-negative outer layers. Teichoic acids consist of 8-50 glycerol or ribitol molecules connected by phosphate ester bridges. Teichoic acid can also vary with the Gram-positive bacteria being investigated. Sometimes teichoic acid can contain erythritol or mannitol or in some instances teichoic acid may be bound to the murein (Schlegel, 1986).

This short study into the outer layers of Gram-negative and Gram-positive bacteria indicates that these outer layers vary in their composition, and variations in composition between bacterial species within these groups is also possible. Such variations in the outer layer composition could change the characteristics of the cell surface to increase or decrease the potential of these bacteria to attach to solid surfaces.

Exterior to the cell wall other bacterial structures can be found, such as capsules, exocellular polymers and pili and fimbriae. It is

important to note that even if capsules and exocellular polymers are present, the components of the cell wall can still be involved in attachment. These cell wall components could be exposed due to the polymer material not covering the bacterium completely, or due to parts of the wall being exposed by cell wall components breaching the polymer wall.

This polymer material is usually polysaccharide in nature, homopolysaccharides such as glucans can sometimes be present, however, heteropolysaccharides such as uronic acid are usually the most common polysaccharides found (Ward et al. 1980). Costerton (1978), suggested that the carbohydrate slimes produced by bacteria were involved in attachment. Other studies have indicated that polymers are critical for the irreversible binding of marine microorganisms to surfaces (Corpe, 1973). Research has shown that many polymers can be produced when bacteria are not attached, therefore, polymers could be involved in other functions other than attachment (Uhilinger, 1983). Mittelman (1984), has shown that one of the other functions of polymers is the binding of copper to bacteria prior to uptake. Many other surface polymers could be involved in the attachment of nutrients to the cell surface prior to transport to the cell.

Polymers have been shown to be affected by the nutritional components in the liquid phase. Bacteria attached to a surface in the presence of polymers produced in glucose-limited media, but not with polymers produced in nitrogen-limited media (Brown et al. 1977). Indeed the polymer produced in nitrogen-limited media, appeared to be inhibiting the intial attachment of the bacteria to the surface. This could have been due to attachment being inhibited by physicochemical

reactions such as ionic repulsion between the surface and the polymer, or by the polymer saturating the binding sights on the bacterial surface so that the bacterium was unable to attach to the surface. This situation can be further complicated, as was shown by Fletcher (1972), who indicated that more than one polymer was produced by a bacterium. These polymers were seen to differ in both their appearance and their function. Marshall (1989) has also demonstrated that the polymers produced during the attachment of Pseudomonas to solid surfaces can differ in structure and so could differ in their function.

This conflicting evidence questions the importance of polymers in relation to attachment. Research into the importance of electrostatic interactions on the attachment of bacteria to solid surfaces suggests that for a bacterium to enter into irreversible attachment, the bacterium must overcome the repulsion barrier present at the solid surface (Section 1.3.1.(A)). Bacterial external appendages such as pili and flagella may then be important when considering bacterial attachment. These appendages could help the bacteria overcome the repulsion energy at the solid surface and allow the bacterium to enter into irreversible attachment.

1.3.2.(B) Bacterial External Appendages

Bacterial attachment has been studied to a great extent and has provided us with information on the different adhesive mechanisms of bacteria. Experiments using the electron microscope found structures on the surface of bacteria such as pili, genetically coded for by a plasmid, and fimbriae, the genetic code for which is found on the bacterial chromosome (Corpe et al. 1975). Research suggests pili and fimbriae could be involved in attachment. This could be possible due to

their long, straight and thin structure, which would allow them to overcome the electrostatic repulsion barrier at a solid surface interface and so help facilitate adhesion. These structures could act as anchors in the attachment of bacteria to surfaces. Pili have also been shown to contain surface polymers of the bacterial cell which can be involved in adhesion, or could influence such factors as hydrophobicity, which can influence attachment (Fadar *et al.* 1984; Handley *et al.* 1984). Ekback, (1986) has shown pili to be important in the attachment of *E. coli* in urinary tract infections. Other *E. coli* studies have shown that fimbriae can be associated with lectins which can be specific for sugars, and this can bring about haemagglutination (Duguid *et al.* 1957 + 1979). Fimbriae have also been shown to be important in the attachment of *Actinomyces viscosus* to lectins (Heeb *et al.* 1982). This evidence does suggest that pili and fimbriae may be important in attachment and their possible significance to attachment in aqueous habitats cannot be ignored.

The importance of flagella in the attachment to solid substrata has again been indicated (Piette, 1991). Flagella can be involved in overcoming the repulsion barrier at a solid surface interface, and have also been shown to be involved in determining the cell orientation at a surface. Therefore, they could influence the potential of a bacterium to become irreversibily attached (Moore *et al.* 1981). *Hyphomicrobium* attachment can occur at the same pole as the flagellum, after attachment the flagellum is subsesquently lost. During the attachment process the *Hyphomicrobium* produces a polymer at the same pole as the flagellum. It is possible that the flagellum initiates this polymer production, or the flagellum could be acting as a means of transporting the polymer from

the bacterium to the surface (Guentzel *et al.* 1975). Researchers have shown that the loss of flagella results in a decrease in attachment. This was thought to be due to their involvement in polymer transfer to the surface rather than their motility ability (Jones *et al.* 1976). Meadows, (1971) and Lawrence, (1987b) have also studied the importance of flagella in attachment. Again the flagella are seen to be involved in the attachment of the bacterium to the surface.

Hyphomicrobium and *Caulobacter* are oligotrophic colonisers of surfaces. These bacteria have specialised structures which are thought to be involved in attachment. Poindexter, (1981) has shown *Caulobacter* to have a stalk, at the end of this stalk a holdfast material can be present. This holdfast material is again thought to be involved in the attachment of the bacterium to surfaces. Other groups of bacteria such as the gliding bacteria, utilise special methods of attachment, an example of this can be seen when *Flexibacter* species use specialised fibrils to attach to surfaces (Ridgeway, 1975).

These attachment studies show a range of different structures involved in attachment, the significance of these structures in the attachment of bacteria at a solid surface interface is questionable. It has been suggested that the time periods involved in bacterial attachment in aqueous environments are too short to indicate the involvement of some of these structures (Marshall *et al.* 1971b). However, Sjollema (1990) has demonstrated that *Streptococcus* mutants which do not have surface appendages do not attach to surfaces. With non-mutant *Streptococcus* with surface appendages attaching to surfaces. Section 1.3.2 has demonstrated that the outer layers of bacteria can vary significantly and the resultant differences in bacterial surface

characteristics could influence the attachment ability of these bacterial to a solid surface.

1.3.2.(C) Attachment Sequence

The primary colonisers of a surface have been described as being both non-specific and permanent (Marshall, 1980). The initial attachment process involves a bacterium coming into contact with, or close proximity to the surface concerned and then attaching to the surface. The process of bacterial attachment to a solid surface has been investigated by Marshall et al (1971a+b). In these studies Marshall has called the initial stage of permanent adhesion, **reversible attachment**. These bacteria can be observed under the light microscope and appear attached. However, the bacteria can easily be removed from the surface, or may swim away from the surface of their own accord. The next step in this process is called **irreversible attachment**. This involves the physiochemical adsorption of the bacterial cell, or the bacterial surface components onto the solid surface. The interactions that can now occur between the bacterium, or its surface components and the solid surface will result in the attraction or repulsion of the two surfaces (Daniels, 1980). It is at this stage in the attachment sequence that bacterial appendages could aid the bacterium in overcoming the repulsion barrier and enter into irreversible attachment. These interactions will also be influenced by various physiochemical factors as discussed in Section 1.3.I.

This attachment process is not strictly defined as demonstrated by Lawrence (1987a+b), during these studies various other stages in the attachment process were indicated. An initial motile attachment phase was reported where the cells were attached to the surface but were still

motile. The reversible attachment phase was then observed when the cells could be seen to be attached apically with the ability to rotate or detach themselves from the surface. The irreversible attachment phase was thought to be initiated by the transition from apical to longitudinal attachment. In this phase it has been suggested that the bacteria could not detach until at least one cell division had occured. The fourth phase is the growth phase when cells can grow to form a microcolony, however, this does not always occur. The last stage is the recolonisation phase when cells can detach and colonise new areas on the surface or new surfaces. In practice these attachment stages are not always observed, this could be due to the complex nature of the process with a large number of external factors also being involved. This attachment complexity was indicated by the various bacterial movements observed during growth and recolonisation by Lawrence (1987a+b). These bacterial movements included a 'packing manoeuver' and a 'spreading manoeuver' which both resulted in the formation of a monolayer of adjacent cells, a 'shedding manoeuver' when the cells were seen to be attached perpendicular to the surface and were seen to release their daughter cells from this position and lastly a 'rolling manoeuver' observed when the cells were loosely attached and continuously somersaulted across the surface as they grew and divided.

During attachment experiments bacteria can attach to solid surfaces in different ways and by using different attachment mechanisms. The attachment of bacteria will be influenced by the factors discussed in Section 1.3 and possibly by the different conditions present in the liquid environment.

1.3.3 The Liquid Environment

The importance of the liquid environment was demonstrated when conditioning films were disucussed in Section 1.3.II, where dissolved substances in the liquid environment could influence bacterial attachment by absorbing onto the solid surface and changing the surface characteristics. The liquid environment will also influence the bacterial cell present in that environment. Changing nutrient conditions have been shown to influence the attachment of bacteria to solid surfaces (McEldowney, 1986). This was thought to occur due to the changes in the nutrient conditions resulting in changes in the bacterial surface characteristics, which in turn influenced the bacterial attachment to the solid surfaces. Other environmental conditions have also been shown to influence bacterial attachment to solid surfaces. These include pH (Stanley, 1967) and the temperature of the liquid environment (Fletcher, 1977).

This thesis will investigate how changing the nutrient and environmental conditions of the liquid environment influences the attachment of pure cultures of bacteria to solid surfaces. If changing the growth conditions in liquid environments changes the relative levels of bacterial species attaching to a solid surfce, then it is possible that by changing the growth conditions of the liquid environment, the microbial composition of the developing biofilm could be influenced. Therefore, this thesis will also investigate how the attachment of mixed bacterial populations to solid surfaces is influenced by changes in the nutritional and environmental conditions in the liquid environment.

1.4 AIMS

The aim of this thesis is to determine if changing the growth conditions of bacteria in aquatic environments can alter the relative levels of bacterial species attaching to solid surfaces and accordingly the microbial composition of the developing biofilm.

The aim was undertaken by studying biofilm development *in vitro* using batch cultures and continuous cultures and *in situ* using a model system. The different growth conditions studied included the nutritional components of the growth media, these included the carbon concentration and the carbon source used in the growth media. The environmental factors considered included the temperature and the pH of the aquatic habitat being investigated.

CHAPTER TWO

ISOLATION AND CHARACTERISATION OF FRESHWATER BACTERIA

2.1 AIMS

To identify a selection of bacteria isolated from a freshwater lake.

2.2 INTRODUCTION

The bacteria isolated from the freshwater lake were identified on the basis of cell morphology, pigment production and the use of a range of biochemical tests.

2.3 MATERIALS AND METHODS

2.3.1 Sterilisation

Solutions, where appropriate, were sterilised using sterile 0.2 μm cellulose acetate filters (Sartorius). Heat sterilisation was carried out by autoclaving as indicated.

All glassware was pre-sterilised by dry heat at 120°C for 6 hours in an oven.

2.3.2 Media Preparation

Media such as Nutrient agar (Oxoid, England) and Nutrient broth (Oxoid, England) were made up as per manufacturers instructions unless otherwise indicated in the text. The diluent used in the preparation of these media was distilled water unless another diluent is specified in the text.

2.3.3 Isolation

A 10ml water sample was obtained from a freshwater lake (Tocil Lake, University of Warwick, Ordnance Survey Map Reference SP2753, SP4306) in a sterile 20ml universal. Serial dilutions of 1ml of this sample were prepared and 0.1ml of 10^{-1}, 10^{-2} and 10^{-3} dilutions of the water sample

were spread plated onto various selective media. The selective media used included nutrient agar plus 50 μg/ml kanamycin (Flint, 1985) which is selective for Flavobacterium species, and various low nutrient media which were used to isolate oligotrophic bacteria which could grow in low nutrient concentrations (Kuznetsov, 1979; Tomomichi, 1978). Nutrient agar (Oxoid Ltd, England), which is nonselective was used to isolate general heterotrophic. These agar plates were incubated at 15°C for up to 7 days.

Bacterial colonies were randomly selected taking note of the colony morphology, pigmentation and colony size. This information was needed for the bacterial identification. The different colony types were then removed and streaked onto nutrient agar plates to purify them ready for their identification.

2.3.4 Identification

(1) Gram stain and cell morphology.

Bacteria grown on nutrient agar (Section 2.3.1) were Gram stained after heat fixing onto glass microscope slides using Jensen's method as described in Cruickshank et al, 1979. They were then examined under a light microscope (Kyowa Optical, model Unilux) at a magnification of X1000 for their Gram reaction and cell morphology.

(2) Bacterial motility.

To observe bacterial motility in the aqueous phase, bacteria were grown in nutrient broth at 15°C in a shaking incubator (150 rpm) for 16 hours. A drop of culture was then placed on a microscope slide and a cover slip was placed on top. This was examined under phase contrast at X400 magnification. The ability of a bacterium to be motile on a solid

surface could also be seen during the initial isolation on nutrient agar plates (Section 2.3.1).

(3) Oxidase Test.

A single colony from a pure culture was picked from a nutrient agar plate with a sterile glass rod and streaked onto a filter paper impregnated with a 1% (w/v) solution of tetramethyl-p-phenylene diamine hydrochloride (Sigma, Poole), in sterile distilled water. A positive violet colour within 5-10 secs indicated cytrochrome oxidase activity (Kovacs, 1956). Pseudomonas fluorescens was used as a positive control, and Escherichia coli as a negative control.

(4) Catalase Test.

A pure colony was picked from a nutrient agar plate with a sterile wooden toothpick and emulsified into hydrogen peroxide (Fisons, Loughborough) solution (30% v/v in distilled water) on a glass slide. The immediate production of bubbles of oxygen indicated a positive reaction and therefore the presence of the enzyme catalase (MacFaddin, 1976).

(5) Hugh and Leifsons Test.

This test is used to differentiate between bacteria possessing fermentative and oxidative metabolic pathways. A basal medium was prepared containing 2.0g peptone (Oxoid Ltd, England), 5.0g NaCl (Fisons, Loughborough), 0.3g K_2PO_4 (Fisons, Loughborough), 0.2g agar (No2, Lab M, London) and 0.0025% (w/v) bromocresol purple (Maynard & Baker Ltd, London) in 1000ml of distilled water, adjusted to pH 7.1 with 1M NaOH. After autoclaving (121°C, 10 min) and cooling, a 1% (w/v) solution of filter sterilised glucose solution was added. The medium was poured aseptically into sterile test tubes. These tubes were stab

inoculated with pure cultures of each organism, half of the tubes were sealed with paraffin oil and all of the tubes were incubated for up to 15 days at 15°C. The production of a yellow colour in the open tubes indicated the oxidation of glucose, a yellow colour in the sealed tubes indicated the fermentation of glucose (Hugh & Leifson, 1953).

(6) Indole Reaction.

The indole reaction demonstrates the ability to break down tryptophan. The test solution consisted of 1g of peptone (Oxoid Ltd, England), 5g NaCl (Fisons, Loughborough) in 1000ml distilled water adjusted to pH 7.4, supplemented by 1% (w/v) tryptophan (Sigma, Poole). The solution was put into tubes in 3ml portions and sterilised by autoclaving (121°C for 15 mins). The inoculated broths were incubated at 15°C until the culture had grown to an optical density of > 0.1 at 540nm as determined by a colorimetry. The formation of indole was detected by the development of a red colour upon the addition of 0.5ml of Kovacs reagent. Kovacs reagent contained 10g p-dimethylaminobenzaldehyde (Sigma, Poole) dissolved in 150ml butyl alcohol (Fisons, Loughborough) with the addition of 50ml concentrated HCl (BDH Chemical Ltd, Poole), (MacFaddin, 1976).

(7) Methyl Red Test.

This test is to detect the production of acid during the fermentation of glucose and that a pH of at least 4.5 is reached after prolonged incubation. The medium used was glucose phosphate peptone water which consists of 5.0g peptone (Oxoid Ltd, England) and 5.0g K_2HPO_4 (Fisons, Loughborough) in 1000ml of distilled water. This medium was dispensed into test tubes in 5ml amounts and sterilised at 121°C for 15 mins. A 10% (w/v) glucose (Fisons, Loughborough) solution was

sterilised by filtration and 0.25ml was added to each tube. This medium was inoculated from a young agar slope culture and incubated at 37°C for 48 hrs. Five drops of methyl red indicator were added to the tubes which were mixed and colour recorded. The methyl red indicator solution consists of 1.0g methyl red (Fisons, Loughborough) and 300ml ethanol in 200ml of distilled water. Positive tubes were bright red and negative tubes were yellow (Cruickshank, 1974).

(8) Voges-Proskauer Test.

Acetyl methyl carbinol can be produced by certain bacteria on the fermentation of carbohydrates or its reduction product 2,3 butylene glycol may be produced. The production of acetyl methyl carbinol or 2,3 butylene glycol usually results in insufficient acid accumulating during fermentation to give a methyl red positive reaction, so the tests were done in conjunction.

The medium used was glucose phosphate peptone water as for methyl red test. The inoculated medium was incubated at 37°C for 48 hrs. After incubation 1ml of 40% (w/v) potassium hydroxide and 3ml of a 5% (w/v) solution of α-naphthol in absolute ethanol were added. A positive reaction was indicated by the development of a pink colour in 2-5 mins, becoming crimson in 30 mins. The tube was shaken at intervals to ensure maximum aeration.

(9) Citrate Utilization Test.

The ability of an organism to utilize citrate as the sole carbon and energy source for growth and ammonium as the sole source of nitogen was investigated in this test. Simmons' citrate agar was used and consisted of 5.0g NaCl (Fison, Loughborough), 0.2g $MgSO_4$. $7H_2O$ (Fisons, Loughborough), 1.0g KH_2PO_4 (Fisons, Loughborough), 5.0g Sodium citrate

(Fisons, Loughborough), 20g agar (No.1 Oxoid Ltd, England) and 40ml of 0.2% (w/v) Bromothymol blue (Sigma, Poole) in 1000ml of distilled water, adjusted to pH 6.8, dispensed into universal tubes and sterilized by autoclaving at 121°C for 15 mins. A positive result was development of a blue colour about the streak, a negative result was the original green colour and no growth (Cruickshank, 1974).

(10) Hydrogen Sulphide Production (Combined with Gelatin liquefaction).

Large numbers of bacteria can produce at least small amounts of hydrogen sulphide from sulphur-containing amino acids. This method was used as it is suitable for differentiation of groups within the Enterobacteriaceae. Hydrogen sulphide production was demonstrated by its ability to form insoluble black ferrous sulphide. The medium was solidified with gelatin rather than agar so it allowed gelatin liquefaction to be tested at the same time.

The medium consisted of 7.5g meat extract (Oxoid Ltd, England), 25g peptone (Oxoid Ltd, England), 5g NaCl (Fisons, Loughborough) and 120g gelatin (Fisons, Loughborough) in 1000ml of distilled water. The pH of the medium was adjusted to 7.6, and this solution was heated in a autoclave in free steam for 10 mins and then at 115°C for 10 mins. The solution was removed from the autoclave as quickly as possible and cooled to about 55°C. Freshly prepared ferrous chloride solution was filter sterilized and 5ml of a 10% (w/v) solution was added to the medium. The medium was placed in narrow tubes and sealed with corks impregnated with paraffin wax and left to cool until the gelatin solidified.

The tubes were inoculated with a straight wire to a depth of 1cm and incubated at 20°C for at least 7 days. Inspection took place daily for blackening due to hydrogen sulphide and for the liquefaction of gelatin.

(11) Fermentation Tests.

The bacterial species to be characterized were tested for their ablity to ferment a range of sugars producing acid, or acid and gas in the process. The basal medium used was peptone water which consisted of 10g peptone (Oxoid Ltd, England) and 5g NaCl (Fisons, Loughborough) in 1000ml of distilled water. The ingredients were dissolved and the pH adjusted to 7.2. After filtration (Whatman No.1) 25ml of a 2% (v/v) solution of Bromocresol indicator (Fisons, Loughborough) was added to 950ml peptone water. This medium was then dispensed in 5ml amounts into test-tubes and autoclaved at 121°C for 10 mins. Durham tubes were introduced to test for gas production.

After the basal medium was cooled the various filter-sterilised carbon sources were added to the basal medium to a final concentration of 0.5% (w/v). The carbon sources were glucose (Fisons, Loughborough), maltose (Fisons, Loughborough), lactose (Fisons, Loughborough), arabinose (BDH Chemicals Ltd, Poole), xylose (Sigma, Poole), inositol (Sigma, Poole), adonitol (Sigma, Poole), malonate (Sigma, Poole) and sucrose (Sigma, Poole).

The various media were inoculated and incubated at 15°C. Some quickly-growing bacteria failed to ferment a given sugar in 24 hrs, but gave a fermentation if incubation is prolonged. Therefore, tests were inspected daily and the day that fermentation first appeared was noted.

A positive fermentation was indicated by a change in colour of the indicator from purple to yellow. Gas production was shown by the presence of gas in the Durham tube.

(12) Urease Activity.

The medium used to demonstrate the hydrolysis of urea consisted of a basal medium consisting of 5g Nacl (Fisons, Loughborough), 2g K_2HPO_4 (Fisons, Loughborough), 1g Peptone (Lab, M), 20g agar (Oxoid Ltd, England) and 0.2g (w/v) phenol red (Sigma, Poole) in 1000ml distilled water. This medium was adjusted to pH 6.8 and autoclaved in a flask at 121°C for 30 mins. The medium was cooled to about 50°C, 100ml 0f a 20% (w/v) solution of urea and 10ml of a 10% (w/v) solution of glucose were filter sterilized and added to the medium. The medium was pipetted aseptically into test-tubes and set as slopes. The slopes were inoculated and incubated at 15°C for up to 72 hrs. The production of a red colour indicated urease activity (Blazevic & Grace, 1975; MacFadden, 1976).

(13) Pigment Production.

The production of pigment by Pseudomonas species was shown using King's media (King, 1954). These media were used to determine if the isolates produced pyocyanin or fluorescin pigments. King's A medium, for enhanced pyocyanin production, consisted of 20g protease peptone (Difco Lab, Michigan), 10g glycerol (Fisons, Loughborough), 10g K_2SO_4 anhydrous (BDH Chemicals Ltd, Poole), 1.4g $MgCl_2$ anhydrous (May & Baker Ltd, London) and 20g agar (No.2, Lab M, London) in 1000ml of distilled water adjusted to pH 7.2. The medium was sterilised by autoclaving at 121°C for 10 mins. Bacteria were streaked onto plates of this medium and incubated at 15°C for 72 hrs. The cells were harvested with 10ml of

chloroform (J. Burrough Ltd, London) and slightly acidified by the addition of 1M HCL (May & Baker Ltd, London). The production of a red colour indicated the presence of pyocyanin typical of Pseudomonas aeruginosa.

King's B medium enhances the production of fluorescin. The medium consists of 20g protease peptone (Difco Lab, Michigan), 10g glycerol (Fisons, Loughborough), 1.5g K_2HPO_4 (Fisons, Loughborough), 1.5g $MgSO_4.7H_2O$ (Fisons, Loughborough) and 20g agar (No.2 Lab M, London) dissolved in 1000ml of distilled water adjusted to pH 7.2. The plates were streaked and incubated at 15°C for 72 hrs. After incubation the colonies present were observed under ultra-violet illumination for yellow-green fluorescence which indicated fluoresein production.

(14) Dihydrolase and Decarboxylase Activity.

The basal medium used to investigate Arginine dihydrolase, Lysine decarboxylase and Ornithine decarboxylase activities of the bacterial isolates was introduced by MacFaddin in 1976. The medium consisted of 0.5g peptone (Oxoid Ltd, London), 5g beef extract (Difco Labs, Detroit), 0.005g cresol red (Sigma, Poole), 5.0mg pyridoxal (Sigma, Poole) and 0.5g glucose (Fisons, Loughborough) in 1000ml distilled water adjusted to pH 6.0. The basal medium was divided equally into four containers and treated as follows:

(a) Add 1% (w/v) L(+) Lysine dihydrochloride (Sigma, Poole).

(b) Add 1% (w/v) L(+) Arginine monodihydrochloride (Sigma, Poole).

(c) Add 1% (w/v) L(+) Ornithine dihydrochloride (Sigma, Poole).

(d) No additives (Control).

These media were then filter sterilised and 3ml aliquots were aseptically dispensed into 5ml test tubes. These tubes were inoculated

and incubated at 15°C for up to 15 days. A positive reaction, that is decarboxylase or dihydrolase activity, was shown by a turbid purple colour.

(15) Polymer Hydrolysis.

The hydrolysis of starch, cellulose, casein and deoxyribose nucleic acid by the bacterial isolates was studied by the methods of Blazovic & Grace (1975) and MacFaddin (1976).

(a) Starch hydrolysis was demonstrated by the addition of 0.8% (w/v) soluble starch (BDH Chemicals, Poole) to nutrient agar (Oxoid Ltd, England). The agar plates were inoculated and incubated at 15°C for 5 days. Starch hydrolysis was demonstrated by flooding the plates with Gram's iodine which consisted of 10g iodine (Fisons, Loughborough) and 20g potassium iodine (Fisons, Loughborough) in 1000ml distilled water. A colourless zone, forming around the bacterial colonies, on medium which stained dark blue indicated a positive result.

(b) To investigate the hydrolysis of cellulose, nutrient agar (Oxoid Ltd, London) containing 10% (w/v) cellulose (Sigma, Poole) was used. The plates were inoculated and incubated at 15°C for 5 days. Cellulase activity was demonstrated by a zone of clearing around the bacterial colonies.

(c) Casein hydrolysis was demonstrated using nutrient agar (Oxoid Ltd, London) containing 10% (w/v) skimmed milk. This milk was sterilised separately and mixed into the nutrient agar on plate pouring. The plates were inoculated and incubated at 15°C for 5 days. Casein hydrolysis was indicated by a zone of clearing around the bacterial colonies.

(d) To investigate DNase activity, DNA agar was used (Oxoid Ltd, London). Plates were inoculated and incubated at 15°C for 5 days. DNA

hydrolysis was shown by a clear zone around the bacterial colonies after flooding the plates with 1M HCl.

(16) Growth on Milk Agar. (10% NaCl).

Milk agar is a good growth medium for staphylococci, however, as staphylococci can tolerate higher concentrations of sodium chloride than other bacteria, salt-containing milk agar is a useful selective medium for staphylococci.

The salt-containing milk agar contained 100ml fresh milk, 200ml sterile nutrient agar containing 3% agar (Oxoid Ltd, London) and 10% NaCl (Fisons, Loughborough). The milk was heated to 60°C shaken and sterilised by autoclaving at 121°C for 20 mins. The sterilised agar was then melted, mixed with the milk and plates were poured. All isolates were streaked onto the agar plates. Positive growth was taken to be a presumptive indication of a Staphylococcus species (Cruickshank, 1979).

(17) Spore Stain.

The spore stain test was used to aid the identification of the Bacillus species. A heat-fixed smear on a glass slide was placed on the rim of a beaker of boiling water, with the bacterial smear uppermost. Once large droplets of water had formed on the under side of slide it was flooded with 5% (w/v) aqueous solution of malachite green (Fisons, Loughborough) for one minute. The slide was removed from the beaker washed with distilled water and stained with 0.05% (w/v) basic fuchin (Fisons, Loughborough) for 30 secs. The slide was washed in distilled water and air dried.

On examination under a light microscope spores were stained green and the bacteria red (Ashby, 1938). A Bacillus megaterium species was used as a positive control.

2.4 RESULTS.

After the initial isolation, the bacterial colonies were purified on nutrient agar. The isolation process for oligotrophic bacteria originally selected for those bacteria which could grow on nutrient-limited media but not on nutrient-rich media. Later recultivation onto nutrient-rich media, however, resulted in growth of these oligotrophic bacteria. According to the classification of Kuznetsov (1979) the bacteria isolated by this process would be bacterial species such as Aeromonas and Corynebacterium and this was observed here. Oligotrophic bacteria sometimes require prolonged incubation periods, and certain species of them may not have been isolated due to the incubation times used in the isolation being too short (Rittman, 1986).

The results obtained from the identification tests (Appendix Tables 1.1-1.10) enabled the bacterial isolates investigated to be tentatively identified, with the aid of Bergey's manual of determinative bacteriology (Table 2.1). The selected species were identified as two Aeromonas species, two Acinetobacter species, two Chromobacterium species, two Pseudomonas aeruginosa, a Pseudomonas fluorescens, a Bacillus species, a Klebsiella species, a Flexibacter species, a coryneform, a Flavobacterium species, an Enterobacter species and a Staphylococcus species.

2.5 DISCUSSION.

The bacteria isolated from Tocil Lake did not in any way reflect the total bacterial population of the lake. The experimental procedures employed were not suitable for the isolation of other bacterial species which could have been present in the lake e.g the oligotrophic bacterium Caulobacter (Poindexter, 1981) and Hyphomicrobium (Staley, 1968). Indeed

TABLE 2.1 Tentative Identification of Organisms Isolated Using Identification Results and Bergey's Manual of Determinative Bacteriology.

ORGANISM	BACTERIA IDENTIFIED
Organism 1	*Acinetobacter* species
Organism 2	*Acinetobacter* species
Organism 3	*Pseudomonas aeruginosa*
Organism 4	*Pseudomonas aeruginosa*
Organism 5	*Pseudomonas fluorescens*
Organism 6	*Chromobacterium* species
Organism 7	*Chromobacterium* species
Organism 8	*Aeromonas* species
Organism 9	*Aeromonas* species
Organism 10	A coryneform
Organism 11	*Flavobacterium* species
Organism 12	*Flexibacter* species
Organism 13	*Bacillus* species
Organism 14	*Staphylococcus* species
Organism 15	*Klebsiella* species
Organism 16	*Enterobacter* species

Yanagita (1978), showed that the numbers of oligotrophic bacteria in freshwater usually exceeds that of copiotrophic bacteria, therefore, this indicates a large number of bacterial species were not isolated by the experimental procedures employed in this isolation.

Research into the effectiveness of the various isolation procedures available, suggests they are not totally effective. Quinn (1984), has used cytochemical techniques for enumeration of metabolically active bacteria and these counts were at least twice as high as heterotrophic plate counts. Other studies have also used activity to enumerate bacteria in a sample and in each case the activity counts were higher than the plate counts (Fry, 1982; Bratbak, 1985). These results indicate that the plate isolation methods employed in this bacterial isolation could only isolate a small fraction of the bacteria present in the lake water sample.

Heterotrophic bacteria were isolated by the methods used. These heterotrophic bacteria were easy to maintain in the laboratory and therefore, the same heterotrophic bacteria could be used in the different attachment experiments. Heterotrophic bacteria have also been used extensively in attachment experiments as indicated by the literature (Marshall, 1977; Fletcher, 1982; McEldowney, 1986). The heterotrophic bacteria isolated were selected for subsequent bacterial attachment experiments in order to make use of the information already available on heterotrophic bacterial attachment in the literature.

The different species of heterotrophic bacteria isolated from Tocil Lake varied with the isolation method used. Certain bacteria had to be isolated on selective media as they needed special growth requirements to grow, without these requirements they would not be competitive on the

other isolation media. The other selective isolation procedures employed e.g. for Flavobacterium were used to isolate these bacteria from a heterotrophic bacterial population which usually out competed them on the nutrient agar used for the isolation of heterotrophs.

The numbers of bacteria obtained and different bacterial species present in a water sample can be influenced by other factors. LeChevallier (1985), has shown that the presence of certain heterotrophic bacteria in a water sample can reduce the numbers of coliform bacteria that are isolated. Bacteria can also be removed from a water sample due to "death" and adsorption to the walls of the sample vessel if the sample is not processed quickly in the laboratory (Cruickshank, 1979). Environmental factors such as seasonal variations in the bacterial populations present in the water samples can occur and so influence the bacterial numbers and species isolated. The water samples in these studies were taken during the winter months and this could have influenced the bacterial species isolated from these samples. These variations can also occur if the sample site is changed or the sample is obtained from the same sample site, but at a different depth (Yoshimzu, 1983; Teweri, 1985). The problem of alltochthonous bacteria (which are not part of the native autochthonous bacterial population) being isolated from the water sample must also be considered. This can arise due to faecal contamination of the water or run off from the soil contaminating the water (Skinner, 1984). This problem can be partly controlled by taking a water sample only after one week without rain, when contamination due to run off will be at a minimum.

On identification the bacterial species identified were predominantly Gram-negative motile rods, however, representatives of

other bacterial groups were also present. The presence of these different heterotrophic bacterial species was important as it enabled the selection of different bacteria species with a range of attachment abilities. These are the bacterial species which would be used in subsequent attachment experiments (Chapter 3).

CHAPTER THREE

AN INVESTIGATION INTO THE ATTACHMENT ABILITIES OF THE BACTERIAL ISOLATES

3.1 AIMS

To select bacteria for further experimental work on the basis of their attachment to glass, in different growth media. Changes in bacterial attachment with subculturing, culture age and bacterial cell concentration were also considered.

3.2 INTRODUCTION

The bacteria, isolated from Tocil Lake, were screened for their ability to attach to glass coverslips in different growth media. Experimental evidence has shown that bacteria can attach differently in different media (McEldowney, 1986).

Changes in bacterial attachment due to storage on solid media or in liquid media and due to subculturing over a period of time were also investigated as culture age and subculturing were known to affect the attachment of bacteria (Fletcher, 1977; Nikaido, 1980).

The literature has indicated that an increase in the bacterial cell concentration used in attachment experiments does not necessarily lead to a increase in the numbers of bacteria attaching to the surface (Fletcher, 1977). Therefore the bacterial cell concentration best suited for the attachment of each bacterial species was also determined.

3.3 MATERIALS AND METHODS

3.3.1 Culture Methods

A pure culture of each of the bacteria isolated from Tocil Lake (Chapter 2), was inoculated into the following media:

(a) Nutrient Broth (Section 2.3.2).

(b) Glucose medium containing 0.544g KH_2PO4, (Fisons, Loughborough), 0.38g NH_4Cl (Fisons, Loughborough), 0.6ml of a salt solution consisting of $10gl^{-1}$ $MgSO_4.7H_2O$ (Fisons, Loughborough), $0.4gl^{-1}$ $FeSO_4.7H_2O$ (Fisons, Loughborough) and 0.2g glucose (Fisons , Loughborough). The glucose and NH_4Cl were prepared in 10ml volumes of distilled water and autoclaved separately at 121°C for 10 mins and added aseptically to the KH_2PO_4 which had been sterilised by autoclaving at 121°C for 15 mins. The salt solution was prepared in 100ml volumes in distilled water, filter sterilised (0.45 μ cellulose acetate filter) and added to the medium to give a final volume of 100ml.

(c) A mannose medium prepared as (b) but with 0.2g mannose (Fisons , Loughborough) instead of glucose.

(d) A sucrose medium prepared as (b) but with 0.2g sucrose (Fisons , Loughborough), instead of glucose.

The inoculated flasks were incubated at 15°C in a shaking incubator for 16 hrs. These flasks were used as stock cultures and stored at 4°C for up to two weeks.

3.3.2 Glass Coverslips

The surfaces used in these experiments were 16mm diameter glass coverslips (Chance Propper Ltd, Warley, England). The coverslips were washed in a mixture of concentrated nitric acid and concentrated sulphuric acid (1/1) for 48 hrs and then rinsed with sterile distilled water six times. After drying, the coverslips were placed in sections of sterile silicon tubing (Watson and Marlow, England) 15mm in diameter and 16mm in length. This silicon tubing enabled the coverslips to be handled more easily.

3.3.3 Absorbance versus Viable Counts

The relationship between absorbance and viable count for each bacterium in Section 3.3.1 was determined in the various media used in this section to aid in the estimations of bacteria present in a flask during attachment experiments. The relationships between absorbance and viable counts of these bacteria in nutrient broth were determined by inoculating 1ml of each stock solution into 100ml of nutrient broth. These cultures were then incubated at 15°C in a shaking incubator at 150 rpm. Growth was followed initially turbidometrically at 540 nm using a Corning colorimeter 252 and by doing viable counts before each colorimeter reading. An identical procedure was used to determine the growth rates of each bacterium used in attachment experiments on each of the media used.

3.3.4 Attachment Experiments

Flasks were set up as in section 3.3.1 and 1ml of a stock culture was aseptically inoculated into each flask. These flasks were incubated at 15°C until late stationary phase was reached. Four coverslips were aseptically added to each flask and the flask was incubated in a shaking incubator for 2 hrs at 150 rpm. After incubation, the coverslips were removed and rinsed in sterile 0.1M phosphate buffer (pH 7.4) or sterile nutrient broth to remove any loosely attached bacteria. The coverslips were placed in Bouin's fixative for 30 secs, rinsed in sterile distilled water and stained with crystal violet (0.5% w/v) (Fisons, Loughborough). The coverslips were rinsed once more in sterile distilled water and left to dry at room temperature. The coverslips were mounted on glass microscope slides and examined under oil immersion using a light microscope, at a magnification of X1000.

The bacterial population remaining in the flask was determined by doing a viable count on nutrient agar plates.

3.3.5 Detachment

Sometimes it was impossible to count the bacteria on a surface due to the presence of large numbers of cells on the surface. Also if there was more than one cell type present, these could not always be distinguished accurately and therefore a microscopic count of the different bacteria present could not always be obtained. In such cases, the bacteria were counted by removal from the surface. To remove the cells, surfactants were used which have been shown to remove bacteria from surfaces under certain conditions (Ball, 1986). The surfactants used were EGTA (Ethylene Glycol-bis (β-aminoethyl Ether) N,N,N,N-Tetra-acetic Acid) (Fisons, Loughborough) and Tween-20 (Fisons, Loughborough). These surfactants were first tested on biofilms prepared using bacteria selected for future attachment experiments to determine their efficiency at removing these bacteria from surfaces.

Once attachment experiments had been performed on glucose media (Section 3.3.4) using selected Gram-negative and Gram-positive bacteria, countable biofilms were obtained. These biofilms were then subjected to various experimental procedures to test which surfactant was best suited for the detachment of these bacteria from the surfaces. After the surfaces were washed to remove loosely attached cells, the surfaces were placed in universals containing 200, 100, 50, and 25 ppm Tween 20 or 20, 10, 5, and 1mg EGTA in a total volume of 5ml sterile distilled water. These universals were incubated at 15°C or 37°C for 30, 60, and 90 mins in shaking incubators at 50, 100, 150, and 200 rpm. The number of detached cells was determined by viable counts. The surfaces themselves

were fixed and stained to determine the number of cells still on the surface (Section 3.3.4). During the detachment process, bacterial cells sometimes became non-viable and this had to be accounted for when the detachment percentages were determined.

Once the best detachment method was obtained the process was performed on each bacterial isolate selected for future attachment experiments, to assess the percentage of detachment under these conditions. Detachment experiments were performed on biofilms grown on nutrient broth and glucose medium (Section 3.3.1) to assess if the nutrient conditions affected the detachment of the bacteria.

3.3.6 The Effects of Subculture on Bacterial Attachment

The bacteria isolated in Section 2.3.1 were subcultured on nutrient agar plates and glucose agar plates (Section 2.3.3) and incubated at 15°C. Stock cultures of each bacteria were prepared (Section 3.3.1) and attachment experiments were performed as in section 3.3.4 using glucose medium and nutrient broth. This procedure was repeated six times to determine the attachment abilities of each isolate after each subculture.

The results obtained from the subculture attachment experiments were expressed as a proportion of the total bacteria attached against the total bacteria present in the flask,

$$\frac{\text{ATTACHED CELLS PER VIEW (63208) } (X_1)}{\text{TOTAL CELLS PRESENT IN FLASK } (X_2)} \pm \sqrt{\frac{X_1^2}{X_2^2}\left(\frac{V(X_1)}{\bar{X}_1^2} + \frac{V(X_2)}{\bar{X}_2^2}\right)}$$

The number of fields of view present during the attachment experiments with four surfaces were 63208, obtained by determining the area of the microscopic field of view (πr^2), dividing this number into the area of the glass coverslip (πr^2) and doubling this number as there were two

sides to the glass coverslips. The results from future attachment experiments were also expressed in this form.

3.3.7 The Effect of Culture Age on Bacterial attachment

Bacterial isolates not selected for future experiments (Section 3.3.4) were inoculated into nutrient broth (Section 2.3.1) and glucose medium (Section 3.3.1). These flasks were incubated at 15°C until stationary phase was reached in an orbital shaker incubator and stored at 4°C. Nutrient agar and glucose agar plates (Section 2.3.3) were streak inoculated with these bacteria and these plates were incubated at 15°C in a standing incubator until full growth was obtained.

Attachment experiments were performed on the bacteria from each of the storage media (Seciton 3.3.4), in the cases of the solid medium the isolates were first grown up in the corresponding liquid medium to enable the attachment experiments to take place. The bacterial population in each flask was estimated by viable count, and these experiments were repeated every seven days.

3.3.8 The Effect of Bacterial Concentration on Attachment

The bacteria selected in Section 3.3.4 were inoculated into 10 nutrient broth flasks and 10 glucose medium flasks (Section 3.3.1). The flasks were incubated at 15°C until stationary phase was reached. The bacteria in the flasks were diluted with sterile nutrient broth or sterile glucose medium to obtain flasks containing different bacterial concentrations in 100ml. Attachment experiments were performed on each flask (Section 3.3.4) and the concentration of bacteria present in each flask was estimated by viable count. These experiments were performed to obtain the bacterial cell concentration which gave the optimum bacterial attachment values.

3.4 RESULTS

3.4.1 Attachment Abilities of Isolates

The results obtained from initial attachment experiments (Table 3.1) indicated no relationship between the attachment of Gram-negative and Gram-positive bacteria, e.g. all the bacteria attached differently to the glass coverslips. There was no relationship between generic classification and extent of their attachment, e.g. Pseudomonas (1) attaches differently from Pseudomonas (3). Similar results have been reported in the literature (Caldwell, 1986; Hsieh, 1986; Zvyagintsev, 1979).

There were variations in bacterial morphology observed, e.g. Flavobacterium became elongated in nutrient broth medium and the Bacillus species was reduced in size in the glucose medium. These phenonemon have been reported in other studies (Caldwell, 1986; Marshall, 1980). These results were considered when selecting the bacteria to be used in the subsequent attachment experiments. The object was to obtain bacteria which varied in their ability to attach to the glass coverslips under different nutrient conditions. This was an important bacterial trait to be investigated in subsequent attachment experiments. Bacteria were not selected if they changed morphology during attachment experiments as this would have complicated their identification on attachment.

3.4.2 Detachment

The results obtained from the numerous detachment experiments (Appendix Tables 2.1-2.24) indicate that the best detachment of the Gram-negative and Gram-positive bacteria was obtained with Tween 20 at 50 ppm at 37°C at 150 rpm in a shaking incubator for 60 mins. This

TABLE 3.1 Attachment abilities of the bacterial isolates under different nutrient conditions.

BACTERIA	NUTRIENT BROTH	GLUCOSE MEDIUM	MANNOSE MEDIUM	SUCROSE MEDIUM	MORPHOLOGY CHANGE
Acinetobacter (1)	-	-	-	-	NO
Acinetobacter (2)	-	-	-	-	NO
Pseudomonas (1)	+++	+	++	+	NO
Pseudomonas (2)	+++	+	++	+	NO
Pseudomonas (3)	+++	+++	+++	+	NO
Chromobacterium (1)	+++	++	++	+	NO
Chromobacterium (2)	+++	++	++	+	NO
Aeromonas (1)	++	++	++	++	NO
Aeromonas (2)	++	++	++	+	NO
Coryneform	++	++	+	-	NO
Flavobacterium	++	+	-	0	YES
Flexibacter	++	++	+	+	NO
Bacillus	++	+	+	0	YES
Staphylococcus	++	+	+	-	NO
Klebsiella	+++	+++	+	0	YES
Enterobacter	++	+	+	+	NO

KEY

- NO ATTACHMENT

+ 0-24% ATTACHMENT

++ 25-59% ATTACHMENT

+++ 60-100% ATTACHMENT

0 NO GROWTH IN MEDIUM

1. The bacteria investigated were grown in and attached in the medium indicated.

TABLE 3.2 The detachment of selected bacteria by 50 ppm Tween 20 in different media in a rotary incubator at 150 rpm at 37°C.

BACTERIA	PERCENTAGE OF DETACHMENT	
	NUTRIENT BROTH	GLUCOSE MEDIUM
Pseudomonas (1)	63.8 (4.9)	83.9 (5.3)
Chromobacterium (1)	86.4 (6.1)	78.6 (4.3)
Aeromonas (1)	80.5 (5.6)	81.8 (5.9)
Coryneform	81.6 (6.3)	66.2 (4.1)
Staphylococcus	55.2 (3.5)	63.6 (4.0)

1. Figures parentheses represent standard error.

2. On detachment cells were viable.

TABLE 3.3 The optimum bacterial concentrations for attachment in nutrient and glucose medium.

BACTERIA	OPTIMUM CELL CONCENTRATION (cfu.ml)	
	NUTRIENT BROTH	GLUCOSE MEDIUM
Pseudomonas (1)	2.1×10^{8}	2.5×10^{8}
Chromobacterium (1)	1.3×10^{8}	0.8×10^{8}
Aeromonas (1)	3.2×10^{8}	2.8×10^{8}
Coryneform	0.7×10^{8}	0.9×10^{8}
Staphylococcus	4.3×10^{8}	3.2×10^{8}

surfactant and the detachment conditions were then used on each of the selected bacterial isolates. The percentage of detachment observed varied with the bacterial species and the growth conditions used (Table 3.2), e.g. the percentage of detachment for Pseudomonas (1) in nutrient broth varied from the detachment value obtained in the glucose medium. It was therefore necessary to obtain a separate detachment percentage each time a bacterium was attached under different conditions in case the percentage of detachment varied with the bacterium or the media used.

3.4.3 The Effect of Subculture on Bacterial Attachment

The results from the subculture experiments (Tables 3.4 + 3.5) were expressed as proportions of the total bacteria present in the flasks. Differences were found in the attachment abilities of all the bacterial isolates after subculture on nutrient agar and glucose agar plates. These differences in attachment continued until the fifth or sixth subculture was performed, e.g. Pseudomonas (2) decreases in its attachment ability after subculturing on nutrient agar or glucose agar plates (Tables 3.4 + 3.5). No statistical analysis was performed on the results obtained in this chapter. The different attachment experiments performed were to give an indication of what factors could influence bacterial attachment and therefore had to be controlled in subsequent attachment experiments.

3.4.4 The Effect of Culture Age on Bacterial Attachment

The data obtained from the studies of the effects of culture age on bacterial attachment ability are variable. If the bacteria were stored at 4°C in liquid media, there were changes in the attachment abilities of these bacteria in subsequent attachment experiments (Tables 3.6 +

TABLE 3.4 The attachment of selected bacteria in nutrient broth after subculture on nutrient agar.

SUBCULTURE	ATTACHMENT AFTER SUBCULTURE		
	Pseudomonas (2)	Chromobacterium (2)	Aeromonas (2)
1	0.3382 (0.0268)	0.3831 (0.0216)	0.3087 (0.0369)
2	0.1472 (0.0234)	0.1664 (0.0255)	0.2721 (0.0306)
3	0.0528 (0.0086)	0.1184 (0.0184)	0.2147 (0.0321)
4	0.0497 (0.0062)	0.1081 (0.0096)	0.1593 (0.0112)
5	0.0518 (0.0078)	0.0984 (0.0119)	0.1310 (0.0148)
6	0.0498 (0.0049)	0.1046 (0.0125)	0.1294 (0.0147)

TABLE 3.4 (Continued)

SUBCULTURE	ATTACHMENT AFTER SUBCULTURE	
	Bacillus	Enterobacter
1	0.0936 (0.0117)	0.0906 (0.0098)
2	0.0559 (0.0068)	0.0513 (0.0063)
3	0.0417 (0.0046)	0.0563 (0.0064)
4	0.0384 (0.0044)	0.0531 (0.0059)
5	0.0371 (0.0049)	0.0536 (0.0061)
6	0.0365 (0.0043)	0.0524 (0.0060)

1. Acinetobacter did not attach to the surfaces in any of these subculture experiments.

2. Figures parentheses represent standard error.

TABLE 3.5 The attachment abilities of selected bacteria after subculture on glucose agar and attachment in glucose medium.

SUBCULTURE	ATTACHMENT AFTER SUBCULTURE		
	Pseudomonas (2)	Chromobacterium (2)	Aeromonas (2)
1	0.2831 (0.0307)	0.1926 (0.0229)	0.2284 (0.0198)
2	0.1269 (0.0134)	0.1317 (0.0186)	0.0962 (0.0098)
3	0.0719 (0.0077)	0.0956 (0.0105)	0.0832 (0.0091)
4	0.0518 (0.0060)	0.0935 (0.0113)	0.0846 (0.0094)
5	0.0467 (0.0053)	0.0885 (0.0094)	0.0685 (0.0072)
6	0.0443 (0.0050)	0.0869 (0.0098)	0.0705 (0.0078)

TABLE 3.5 (Continued)

SUBCULTURE	ATTACHMENT AFTER SUBCULTURE	
	Bacillus	Enterobacter
1	0.2436 (0.0245)	0.1158 (0.0127)
2	0.2015 (0.0214)	0.0768 (0.0081)
3	0.0963 (0.0112)	0.0532 (0.0059)
4	0.0428 (0.0053)	0.0204 (0.0032)
5	0.0439 (0.0057)	0.0229 (0.0029)
6	0.0426 (0.0050)	0.0218 (0.0025)

1. Acinetobacter did not attach to the surfaces in any of these subculture experiments.

2. Figures in parentheses represent standard error.

TABLE 3.6 The attachment abilities of selected bacteria after storage and attachment in nutrient broth.

TIME (WEEKS)	ATTACHMENT AFTER STORAGE Pseudomonas (2)	Chromobacterium (2)	Aeromonas (2)
0	0.0526 (0.0054)	0.1250 (0.0127)	0.1387 (0.0153)
2	0.0647 (0.0074)	0.1067 (0.0120)	0.1738 (0.0172)
4	0.0636 (0.0061)	0.1572 (0.0180)	0.1834 (0.0175)
6	0.0439 (0.0046)	0.1158 (0.0098)	0.1737 (0.0171)
8	0.0511 (0.0053)	0.1318 (0.0140)	0.1443 (0.0142)

TABLE 3.6 (Continued)

TIME (WEEKS)	ATTACHMENT AFTER STORAGE Bacillus	Enterobacter
0	0.0372 (0.0041)	0.0538 (0.0059)
2	0.0558 (0.0062)	0.0513 (0.0050)
4	0.0339 (0.0038)	0.0627 (0.0068)
6	0.0418 (0.0046)	0.0569 (0.0064)
8	0.0426 (0.0045)	0.0597 (0.0063)

1. Acinetobater did not attach to the surfaces in any of these storage experiments.

2. Figures in parentheses represent standard error.

TABLE 3.7 The attachment abilities of bacteria after storage and attachment in glucose medium.

TIME (WEEKS)	ATTACHMENT AFTER STORAGE		
	Pseudomonas (2)	Chromobacterium (2)	Aeromonas (2)
0	0.0447 (0.0051)	0.0914 (0.0095)	0.0764 (0.0071)
2	0.0561 (0.0060)	0.0712 (0.0076)	0.0883 (0.0092)
4	0.0329 (0.0041)	0.1034 (0.0114)	0.0651 (0.0069)
6	0.0459 (0.0053)	0.0793 (0.0086)	0.0895 (0.0092)
8	0.0531 (0.0058)	0.0912 (0.0110)	0.0652 (0.0062)

TABLE 3.7 (Continued)

TIME (WEEKS)	ATTACHMENT AFTER STORAGE	
	Bacillus	Enterobacter
0	0.0453 (0.0048)	0.0225 (0.0030)
2	0.0591 (0.0063)	0.0318 (0.0033)
4	0.0437 (0.0047)	0.0275 (0.0027)
6	0.0487 (0.0052)	0.0384 (0.0041)
8	0.0464 (0.0057)	0.0301 (0.0038)

1. Acinetobacter did not attach to the surfaces in any of these storage experiments.

2. Figures in parentheses represent standard error.

3.7). These changes did not correspond with the length of storage, e.g. in nutrient broth the attachment abilities of Pseudomonas (2) and the Enterobacter were relatively constant over the eight week period, however, the attachment of Chromobacterium (2) varied over this eight week period. The results obtained after the bacteria were stored on solid media were less variable and changes in attachment with culture age were only observed after six or eight weeks, e.g. the Bacillus and Pseudomonas (2) attachment ability was reduced after six weeks storage on nutrient agar (Tables 3.8 + 3.9).

The other main problem encountered was the contamination of agar plates during these storage periods, this was also observed with the liquid media. In order to overcome this problem, bacteria were cultured on nutrient agar and glucose plates using the spread plate method which only gave single bacterial colonies on the agar plates which were less prone to contamination. Numerous plates for each isolate were therefore produced and stored at 4°C until required. The isolates were inoculated into the required media and stock cultures were prepared. The attachment abilities of the bacterial isolates were investigated to ensure they had not significantly changed during storage.

3.4.5 The Effect of Bacterial Concentration on Attachment

The isolates tested for the effects of culture concentration on attachment produced different optimum values for the concentration of bacteria which gave no increase in attachment (Table 3.3). The lowest optimal bacterial concentration where no change in bacterial attachment was observed was for the coryneform and therefore it was important to keep the bacterial concentrations below this value in future experiments. This was necessary so that any changes observed in

TABLE 3.8 The attachment abilities of the bacteria after storage on nutrient agar and attachment in nutrient broth.

TIME (WEEKS)	ATTACHMENT AFTER STORAGE		
	Pseudomonas (2)	Chromobacterium (2)	Aeromonas (2)
0	0.0486 (0.0045)	0.0976 (0.0099)	0.1275 (0.0119)
2	0.0473 (0.0051)	0.1049 (0.0115)	0.1247 (0.0122)
4	0.0457 (0.0049)	0.1028 (0.0098)	0.1158 (0.0106)
6	0.0421 (0.0046)	0.0859 (0.0092)	0.0945 (0.0097)
8	0.0387 (0.0039)	0.0784 (0.0081)	0.0956 (0.0094)

TABLE 3.8 (Continued)

TIME (WEEKS)	ATTACHMENT AFTER STORAGE	
	Bacillus	Enterobacter
0	0.0382 (0.0040)	0.0559 (0.0059)
2	0.0389 (0.0038)	0.0526 (0.0054)
4	0.0410 (0.0045)	0.0568 (0.0061)
6	0.0339 (0.0037)	0.0562 (0.0058)
8	0.0316 (0.0035)	0.0510 (0.0053)

1. Acinetobacter did not attach to the surfaces in any of these storage experiments.

2. Figures in parentheses represent standard error.

TABLE 3.9 The attachment abilities of bacteria after storage on glucose agar and attachment in glucose medium.

TIME (WEEKS)	ATTACHMENT AFTER STORAGE		
	Pseudomonas (2)	Chromobacterium (2)	Aeromonas (2)
0	0.0448 (0.0042)	0.0856 (0.0095)	0.0698 (0.0069)
2	0.0453 (0.0048)	0.0867 (0.0090)	0.0713 (0.0072)
4	0.0455 (0.0041)	0.0884 (0.0093)	0.0695 (0.0078)
6	0.0419 (0.0043)	0.0805 (0.0087)	0.0523 (0.0060)
8	0.0431 (0.0045)	0.0937 (0.0112)	0.0529 (0.0059)

TABLE 3.9 (Continued)

TIME (WEEKS)	ATTACHMENT AFTER STORAGE	
	Bacillus	Enterobacter
0	0.0439 (0.0048)	0.0215 (0.0026)
2	0.0426 (0.0043)	0.0241 (0.0028)
4	0.0441 (0.0049)	0.0318 (0.0031)
6	0.0384 (0.0042)	0.0312 (0.0035)
8	0.0382 (0.0040)	0.0315 (0.0034)

1. Acinetobacter did not attach to the surfaces in any of these storage experiments.

2. Figures in parentheses represent standard error.

attachment in future experiments due to other experimental factors, could be detected. This optimum cell concentration varied with growth medium, e.g. the optimum cell concentration for Aeromonas (1) in nutrient broth was different from the value obtained in the glucose medium (FIGURE 3.2).

3.5 DISCUSSION

3.5.1 The Bacterial Cell and Attachment

The different bacterial isolates attached differently to the glass coverslips with no relationship between the generic classification and attachment. Similar results were reported in the literature (Zvyagintsev, 1979) and these different attachment abilities must be due to the bacteria varying in other factors such as cell surface characteristics or in physiological activity.

Motile and non-motile isolates attached to the surfaces in these experiments. However, it would be wrong to conclude that motility is not important in the attachment process. In situations, within macroenvironments of flowing systems, the importance of motility to attachment is questionable, however, when considering microenvironments the presence of a hydrodynamic boundary layer could increase the significance of motility in attachment with motility possibily involved in carrying the bacteria against the direction of flow to allow attachment to the surface (Caldwell, 1984). Piette (1990) has suggested that motility indirectly helps bacterial attachment to solid surfaces by increasing the number of bacteria reaching the surface in a given time. This phenomenon is important if the surface is beneficial to the bacterium's growth, e.g. if the surface supplies nutrients for bacterial growth (Fletcher, 1984).

Motility can also result in the bacterium arriving perpendicular to the surface which has been shown to be important in the initial attachment process (Lawrence, 1987a). Motility can also be important once the bacterium has arrived at a surface. After the bacterium has become reversibly attached, it must overcome the repulsion barriers present at the surface to become irreversibly attached (Marshall, 1974; Fletcher, 1984) and it is possible that motility could aid the bacterium in this. Reports have suggested that attachment is an active process facilitated by motility and therefore an increase in attachment could be obtained with bacterial motility (Lawrence, 1987a). It could also be due to motile bacteria coming randomly into contact with the surface more often than non-motile bacteria. There could also be other factors involved indicated by the fact that non-motile cells can also attach to the surfaces like their motile counterparts (Lawrence, 1987b).

There have also been reports of bacteria attaching to solid surfaces by their polar flagella (Meadows, 1971) with the flagellum being the initial point of contact between the bacterium and the solid surface. This interaction could be due to the diameter of the flagellum being smaller than that of the bacterium and possibly allowing a lowering of the repulsion barrier between the bacterium and the solid surface (Rogers, 1979). This initial attachment with the flagellum may not last, and for irreversable attachment, the surface of the bacterium will have to come into contact with the solid surface (Sjoblad, 1982).

As the bacterial surface itself acts as a point of contact between the bacterium and the solid surface, the different cell surface characteristics of the bacterium could be important in the attachment process. Indeed, it has been suggested that the different attachment

levels displayed by bacteria can be attributed to their different cell surface characteristics (McEldowney, 1986). In these studies, different attachment levels were obtained due to the surface components reacting differently at a physiochemical level with the solid surface (Chapter 4).

Physiological activity can also be an important bacterial factor to consider in attachment studies. The effect of this activity on attachment can again vary with the bacterial species involved and it is also possible the physiological activity can influence other important attachment factors such as cell surface characteristics. The physiological activity of a bacterium can itself be affected by factors such as growth conditions (Fletcher 1984). In all the attachment experiments, the cells were grown to early stationary phase, this was necessary as slight changes in the growth stage lead to significant changes in bacterial attachment (Fletcher, 1977; Zvyagintsev, 1973; Minato, 1979). Feldner (1983), demonstrated that bacteria in different growth stages were affected differently by proteins present on the attachment surface. The attachment of early log phase or stationary phase cultures was more inhibited by proteins on the surface than other growth phases. Stationary phase was chosen in these attachment experiments as a constant state, although in practice some species of bacteria may have attached better or worse in other growth phases.

As the aim of subsequent experiments was to investigate attachment under different growth conditions such as carbon source, a brief survey of bacterial attachment in different carbon sources was carried out to select bacteria for these future experiments. The results (Table 3.1), indicated that the bacteria attached differently in the different carbon

sources as was expected from the literature (McEldowney, 1986). Caldwell (1986), used different growth conditions to select for bacteria which could attach to surfaces. This procedure was not used in these studies as the bacteria isolated did not vary significantly in attachment abilities. The bacteria selected for future use were chosen as their attachment abilities to surfaces varied significantly. The bacterial morphology on attachment to surfaces was also studied. Bacterial cells have been shown to decrease in size during attachment (Caldwell, 1986; Marshall, 1980) or elongate after attachment (Lawrence, 1987b). Bacteria which were seen to vary in cell morphology on attachment were not selected for subsequent experiments as these morphology changes could hinder bacterial identification after attachment.

3.5.2 Detachment

The detachment of bacteria from a surface will be influenced by the bacterial species present on the surface, the surface itself and the surfactant used (Scheraga, 1979). During these studies, the detachment rates were not changed by varying the pH or electrolyte concentration used. This was thought to be due to a strong bonding between the bacteria and the surface. As bacteria which are strongly attached are hard to detach, numerous methods of detachment have been tried.

Antimetabolites have been shown to decrease the attachment of a Vibrio species to surfaces. This process usually resulted in the non-attached bacteria being non-viable, therefore, the numbers of these non-attached bacteria obtained would be hard to determine (Paul, 1984). Non-viable non-attached bacteria were also obtained when taurolin, an non-antibiotic antimicrobial agent, was used to reduce the adherence of

bacteria to surfaces. When taurolin was used in sub-minimal inhibitory concentrations, the adherence of E.coli and Canidida albicans was reduced or prevented. To influence the attachment of a Staphylococcus species, much higher concentrations of taurolin were required. This process resulted in the bacterial cells being unable to complete cell division, losing their fimbriae and becoming elongated. Due to the non-attached cells being non-viable and their morphology changing on detachment, this process again made the numbers of non-attached cells hard to determine. The above processes only influenced bacterial attachment before attachment occurs, therefore, were not suited to detach bacteria when they had already attached.

Gollinge (1985), suggested using monoclonal antibodies to detach bacteria from surfaces. Studies indicated that the attachment of Bordetella pertussis was influenced by monoclonal antibodies, however, the detachment of these bacteria by these antibodies was not reported. Degradative enzymes such as pronase and trypsin detached some bacteria from surfaces, the proportion of detachment obtained however, again depended on the surfaces and the species used in the experiments (Corpe, 1974b; Fletcher, 1980; Danielsson, 1977). The attachment of Straptococcus faecium to surfaces was seen to be reversed by the surfactant Tween-80 (Orstavik, 1977). Surfactants are amphiphilic molecules composed of a hydrophobic portion and a charged or polar portion. They include anionic and cationic detergents and non-ionic types. These surfactants have been used by other researchers in detachment experiments with varying degrees of success (Ball, 1986). These surfactants were used in these detachment experiments as at certain concentrations the bacteria could be detached from the surface

and still retain their viability, and therefore the detached bacteria were countable.

Problems can still arise when these surfactants are used, because cell lysis has been reported to be involved in detaching cells from surfaces (Corpe, 1974b). Many external factors such as the length of time of the attachment or detachment (Fletcher, 1977) and the temperature at which the procedure is performed (Berger, 1986) have been shown to influence attachment and possibly detachment.

3.5.3 Experimental Procedure and Attachment

Other variables also have to be controlled during the attachment experiments. During the purification and maintenance of bacterial strains, subculturing is often employed. The results from the subculture experiments show this procedure can greatly affect the attachment ability of the bacterial species. These changes in bacterial attachment could be due to variations in the bacterial cell surface characteristics during the subculturing. Wild type strains of bacteria have been shown to vary in the quantity of LPS on the surface after subculture (Nikaido, 1980). Changes in the LPS can influence the hydrophobic or charge interactions a bacterium can exhibit, and therefore this could influence their attachment (Magnusson, 1977). The evidence from these attachment experiments indicate that the changes observed in attachment ability became insignificant after five or six subcultures. Although the bacterial surface after subculture may be different from that in a natural environment, the use of bacterial cultures which had been subcultured six times was the only way to control the effects of subculture on the attachment of the bacterial isolates, although it is

recognised that subculturing reduced the attachment rate for almost all the species tested.

The results obtained from the effects of culture age on attachment are variable, with changes in attachment being observed after long periods of storage. Reports have suggested that this could be due to changes in bacterial surface components such as polymers on the surface of the cell which could change in composition or their effectiveness with time (Chapter 4). Changes in bacterial attachment with culture age could also be related to motility with a bacterium becoming less motile with age (Fletcher 1977). Microscopic inspection of bacteria revealed that log-phase cultures had greater proportions of motile cells than stationary phase (Fletcher. 1977). Therefore, the reduction in motile cells could account for the differences in attachment obtained in these studies. As explained previously in this chapter, stationary phase cultures only were used in subsequent experiments to control this factor. Heeb (1982), has shown that Actinomyces viscosus could be stored refridgerated for months without significant deterioration in their ability to attach to beads. This suggests that each bacterium's attachment must be studied seperately under storage conditions to determine the changes which occur during storage.

The concentrations of bacteria used in these experiments is important. Experimental evidence indicates that there are optimum cell concentrations above which no more attachment occurs (Gordon, 1983). Carrie (1985) demonstrated that the changing attachment rates of bacteria was directly related to the bacterial concentration, providing that the physiological state of the bacteria remained unaltered. The changes in attachment with bacterial concentration were not surprising,

with an increasing number of bacteria there would be a greater number of collisions between the bacteria and the surface. Therefore, the opportunities for attachment to take place would increase. Attachment reaches a maximum value when the surface attachment sites become fully saturated with bacteria and hence cannot support any more attachment. It is therefore important to work at bacterial concentrations and for time periods which prevent or do not allow this to happen. If saturation occurs within the time period of the experiment, then differences in attachment rate due to the different environmental factors being studied would be obscured because under all conditions maximal rates of bacterial attachment would have been recorded.

In many cases the surface is not fully covered with bacteria, this could be due to parts of the surface not being available for attachment (Doyle, 1982). This could be due to the surface being conditioned in some adverse way (Fletcher, 1982) i.e. with molecules that inhibit attachment, or due to bacteria not attaching to the conditioned surface for some physiochemical reasons. Fletcher (1976), demonstrated that proteins such as fibrinogen and gelatin adsorb onto a surface inhibited the attachment of a Pseudomonas species. The mechanism by which these molecules inhibit attachment vary, depending on the macromolecules present. Dextrans and LPS, although largely polysaccharide in structure, are significantly different and so are the mechanisms by which they inhibit attachment. LPS can inhibit bacterial attachment to a substrata when LPS is added to the liquid phase with the bacterial cells or when LPS is adsorbed onto the substrata before attachment. The dextrans, however, could only inhibit attachment when added to the cells during attachment. This suggests different mechanisms by which attachment is

inhibited (Pringle, 1986). Whenever a solid surface is immersed into an aqueous solution, substances are quickly adsorbed onto the surface. Macromolecules such as proteins, are generally irreversibly adsorbed and tend to mask the original properties of the substratum. The extent to which these macromolecules obscure the original chemistry of the surface is not clear, however, it must be considered during attachment experiments (Baier, 1981).

The attachment abilities of bacteria can be influenced by this conditioning film at the liquid-surface interface. The substratum charge can influence the ions or charged molecules present on the surface and this could influence factors such as pH (Hattori, 1963) and the types of nutrients present on the surface (Marshall, 1976). If large molecules are absorbed onto surfaces, they could change their conformation, becoming more or less accessible to bacteria, and therefore could influence bacterial attachment to these surfaces.

Problems could also occur due to roughness on the surface (Baker, 1984). This could cause an increase in attachment due to a larger surface area being present for attachment or the rough areas could provide a protected and more favourable area for colonisation. The roughness of the surface was beyond experimental control and it was assumed that each surface was affected in the same way. For a single surface like glass, this could be an acceptable assumption but it is accepted that the degree of roughness between surfaces of different composition would be different. Results therefore have to be interpreted with this in mind.

Bright (1983), demonstrated that the ratio between attached and free-living bacteria depended on the substratum used. In his studies,

bacterial activity was more important in the initial attachment of the bacterium to hydrophobic surfaces than to hydrophilic ones. Moreover, different surfaces have been shown to follow a characteristic succession of microorganisms, so that the final biofilm that forms on one surface under controlled conditions differed from that formed on another under the same conditions (Marszalek, 1979: Tamplin, 1990). As the biofilm that develops on different surfaces varies, only one surface, glass, was used in subsequent experiments.

3.5.4 Physico-Chemical Properties of Substratum

The above physical substratum properties are important when considering attachment, however, the chemical properties of the surface must also be considered (Chapter 1). The surface free energy of the substratum is one property to be considered. The surface free energy of the substratum is the surface force capable of interaction with forces in surrounding phases. The free energy of a surface is the energy resulting from the surface groups, molecules or atoms, which are able to interact with other groups, molecules or atoms, which may approach the surface. The molecules within the bulk of the substratum can interact with the surrounding substratum molecules, however, the molecules at the surface of the substrata can only interact with the molecules below them in the bulk phase. Therefore, the molecules available for interaction with other molecules would be greater at the surface, and so the free energy at the surface would be greater than the interior of the surface.

The surface free energy is an important indicator of the suitability of that surface for the attachment of bacteria, as surface free energy indicates the tendency for the substratum to enter into various types of interactions. Surface free energy is also a measure of the degree to

which water can be adsorbed onto the surface. Water can enter into the same interactions as the surface. The interactions involved include;

(a) van der Waals dispersion interactions, which are weak charge interactions resulting from the fluctuating distribution and temporary spatial concentration of electrons in molecules and atoms.

(b) electrostatic interactions, between charged groups.

(c) polar interactions, between groups with permanent or induced dipoles.

(d) chemical bonding, including ionic, covalent and hydrogen bonding.

When considering water and a substratum, high-energy surfaces tend to be hydrophilic with lower energy surfaces tending to be hydrophobic. As with surface free energy the hydrophilicity of the substratum can influence the attachment of bacteria to that substratum (Chapter 1).

CHAPTER FOUR

THE EFFECT OF CARBON SOURCE, CARBON CONCENTRATION, CARBON-TO-NITROGEN RATIO AND TEMPERATURE ON THE ATTACHMENT OF BACTERIA.

4.1 AIMS

To study the effects of different carbon sources, carbon concentrations and carbon to nitrogen ratios on the attachment of bacteria grown in batch culture and continuous culture. The effect of temperature on the attachment of these bacteria was also investigated in this chapter.

4.2 INTRODUCTION

Varying the growth conditions of a bacterium can greatly affect the attachment abilities of that bacterium (Williams. 1977). Carbon source, carbon concentration and the carbon-to-nitrogen ratio of a medium have been found to have a significant effect on the attachment abilities of bacteria, and these effects are also dependent on the strain of bacterium used in the attachment experiments (McEldowney,1986). The different attachment abilities observed, due to changes in the carbon source and the carbon-to-nitrogen ratio, were thought to be related to characteristics of the cell surface. It is well known that changes in growth conditions affect the cell surface (Fletcher, 1982; McEldowney 1986). Therefore, changes in other growth conditions such as the temperature could also influence the cell surface and in return influence bacterial attachment.

Temperature has been shown to affect the attachment of bacteria (Fletcher, 1977; Stanley, 1967; Gong, 1989). Minato (1976) demonstrated how different species of bacteria varied in their ability to attach to surfaces at different temperatures. The differences observed in

bacterial attachment abilities due to temperature were thought to be due to changes in the bacterial physiology or changes in the physico-chemical interactions involved in bacterial attachment (Marshall, 1976).

The carbon and nitrogen concentrations used in the growth media are also important, with high and low carbon concentrations bringing about changes in the attachment abilities of bacteria. Bacteria have been shown to attach to surfaces under low nutrient conditions and this could be beneficial for growth because the bacterium obtains nutrients which pass the surface, with no energy being expended to move the bacterium to the nutrients (Jannasch, 1972). If there is excess nutrients present in the liquid phase, the cells may not attach due to there being sufficient nutrients in the liquid phase for growth and attachment may not be of any benefit to the bacterium. This would be rare in the natural environment where nutrients are usually limiting. In the laboratory however, where continuous culture and batch techniques are used, high nutrient concentrations have often been used in attachment experiments and will therefore be considered.

4.3 MATERIALS AND METHODS

4.3.1 Bacteria and Inoculation

Stock cultures of Aeromonas 2, Chromobacterium 2, Staphylococcus species and the coryneform were set up in the media required for these experiments. Batch cultures were inoculated with 1ml of a stock culture and incubated as in Section 3.3.1 until late stationery phase was reached. 10ml volumes of these stock cultures were used to inoculate the continuous culture apparatus and these continuous culture systems were run at 15°C for 3 days with controlled aeration and a dilution rate of 0.2 h^{-1}. The continuous culture sample was also stirred to ensure good

mixing of the oxygen and nutrients in the vessel (Figure 4.1). Continuous culture systems were run for 5 days.

4.3.2. Culture Conditions

(A) Batch Culture

The basic medium used in these experiments was described in Section 3.3.1. The concentrations of carbon and nitrogen used in the basic medium in these experiments were as follows:

(1)	0.005	g carbon l^{-1}	0.038	g NH_4Cl l^{-1}
(2)	0.02	g carbon l^{-1}	0.002	g NH_4Cl l^{-1}
(3)	0.02	g carbon l^{-1}	0.038	g NH_4Cl l^{-1}
(4)	0.05	g carbon l^{-1}	0.38	g NH_4Cl l^{-1}
(5)	0.2	g carbon l^{-1}	0.02	g NH_4Cl l^{-1}
(6)	0.2	g carbon l^{-1}	0.38	g NH_4Cl l^{-1}
(7)	0.5	g carbon l^{-1}	3.8	g NH_4Cl l^{-1}
(8)	2	g carbon l^{-1}	0.2	g NH_4Cl l^{-1}
(9)	2	g carbon l^{-1}	3.8	g NH_4Cl l^{-1}

The carbon sources glucose, mannose and sucrose used in these experiments and the sterilization procedures employed were as in Section 3.3.1. These concentrations of carbon and nitrogen were chosen as previous bacterial attachment studies had used similar concentrations of carbon and nitrogen in some instances (Fletcher, 1983; McEldowney, 1986). The other concentrations of carbon and nitrogen were used to give a diverse selection of nutrient conditions which could be used to study their effect on bacterial attachment ability.

(B) Continuous Culture

The continuous culture system enabled the temperature, aeration and dilution rate of the culture to be controlled, and hence the growth rate of the culture was at a steady state (FIGURE 4.1). The basic medium used in these experiments consisted of 54.4g KH_2PO_4 (Fisons, Loughborough) in 10 litres of distilled water adjusted to pH 7.4 with 10M NaOH. This medium was then autoclaved at 120°C for 40mins, and then 60ml of a filter-sterilized salt solution (Section 3.31) was aseptically added to the medium. The concentrations of carbon and nitrogen used in the test media were as for media (4), (5) and (6) in the batch experiments (Section 4.3.2.A) and the carbon sources used were sucrose, glucose and mannose (Section 3.3.1).

After inoculation and growth in the continuous culture system, a bacterial sample was removed from the sample bottle (Figure 4.1) and placed into a sterile 100ml flask. The attachment experiments were then performed as for batch cultures.

4.3.3 Attachment Experiments

Four surfaces (16mm diameter glass coverslip) were aseptically added to the batch and continuous culture samples. The attachment experiments were performed in the growth media that were used in these experiments. The flasks were then incubated at 15°C for 2 hours to allow attachment to occur. The surfaces were then removed and treated as in Section 3.3.1 or Section 3.3.5 depending on the complexity of the bacterial biofilm. The solid surfaces in the continuous culture system were taken out after the system was run for 5 days and the surfaces were treated as in Section 3.3.5.

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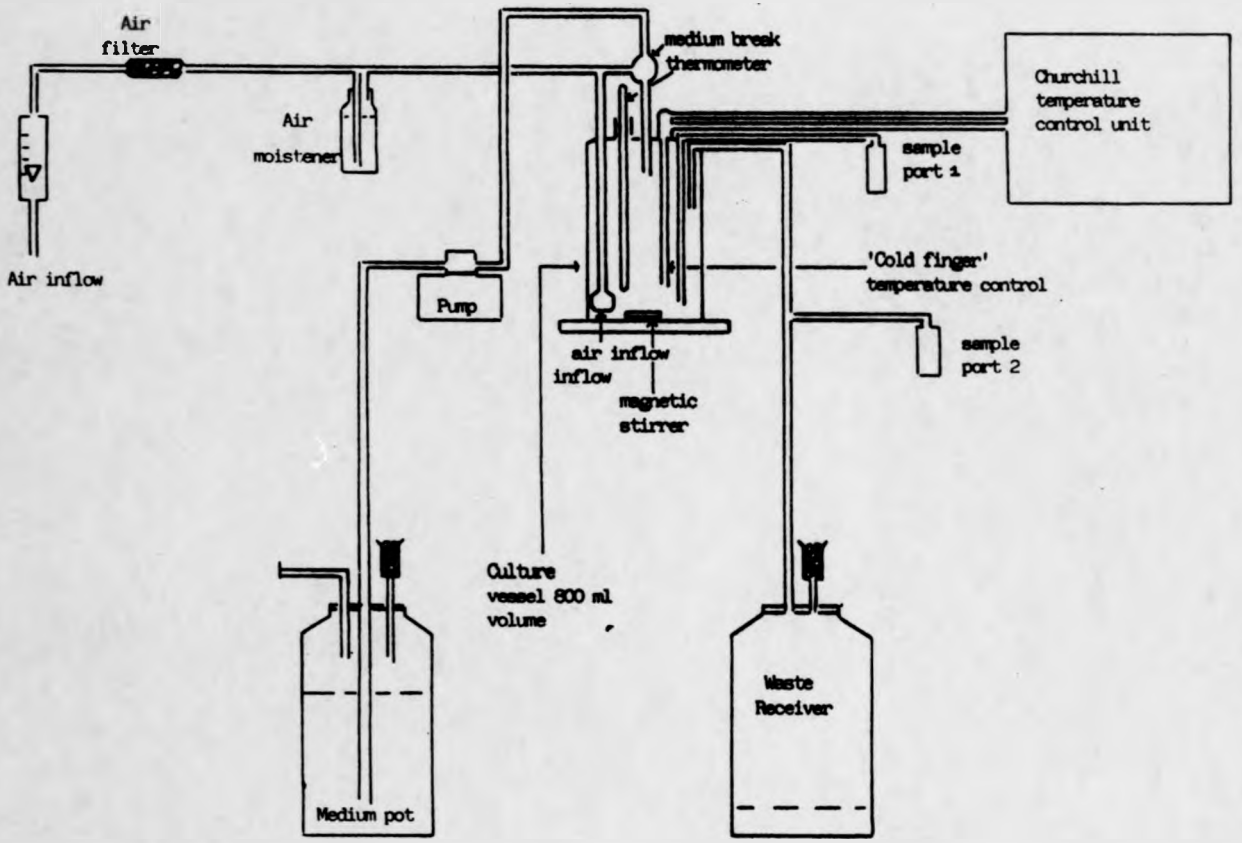

Figure 4.1 The continuous culture system (not drawn to scale)

4.3.4 Effect of Temperature on Bacterial Attachment Ability.

The effect of temperature on bacterial attachment in batch culture was examined using some of the media in Section 4.3.2.(A). The bacteria and media were prepared as in Section 4.3.2.(A). However, only media (4), (5) and (6) with the three carbon sources glucose, mannose and sucrose were used to study the effects of temperature on attachment. These media were used as they would allow comparison of the results with those obtained from the continuous culture attachment experiments which also used media (4), (5) and (6) with the three carbon sources glucose, mannose and sucrose.

The bacteria were prepared, inoculated and incubated at 15°C in batch culture and continuous culture as in Section 4.3.2.(A+B). Three samples of each medium were prepared. The attachment experiments were then performed as in Section 4.3.3, however, the three flasks of each medium were incubated in a shaking incubator at 150 rpm at different temperatures during the bacterial attachment experiments. The temperatures chosen were 15°C, 25°C and 37°C. Once the attachment experimente were completed the surfaces were treated as in Section 3.3.5 to determine the numbers of attached bacteria on the surfaces.

4.3.5 Statistical Analysis

To determine if there was a significant difference between two results obtained in these experiments t-tests were performed.

$$t = \frac{\bar{x} - \bar{y}}{S\sqrt{\frac{1}{nx} + \frac{1}{ny}}}$$

Where x was one attachment value and y was another. The sample sizes (The number of fields of view counted) nx and ny were both 25. The numbers of counts done to obtain x and y. S was obtained by

$$S = \sqrt{SD^2 + SD^2}$$

Where SD is the standard deviation of each sample of field of view counts.

4.4 RESULTS

Appendix FIGURE 1 (a) and (b) gives examples of the types of biofilms obtained during the attachment experiments in this chapter.

4.4.1 Bacterial Attachment in Batch Culture Grown Cells.

The results indicated that growth conditions, whether batch culture or continuous culture, had a profound effect on the attachment of the bacterial species. When the bacteria were grown in batch culture attachment varied with the carbon source, carbon concentration and the carbon-to-nitrogen ratio of the medium used in the experiment. The different bacterial species, however, did not always show the same response to the varying growth conditions (FIGURE 4.2).

The carbon-to-nitrogen ratio could influence the attachment of bacteria but, the effects on attachment observed varied with the bacterial species present and the carbon source used. Aeromonas attached significantly more (P>0.05) in the glucose Medium (5) which was nitrogen-limited rather than the glucose Medium (4) which was carbon-limited. This was not the case, however, when a different carbon source was used during attachment. When the sucrose medium was used, Aeromonas attached to a significantly greater extent (P>0.05) in the carbon-limited Medium (4) compared to the carbon-to-nitrogen-limited Medium (5) (FIGURE 4.2 (a)I + (a)III)).

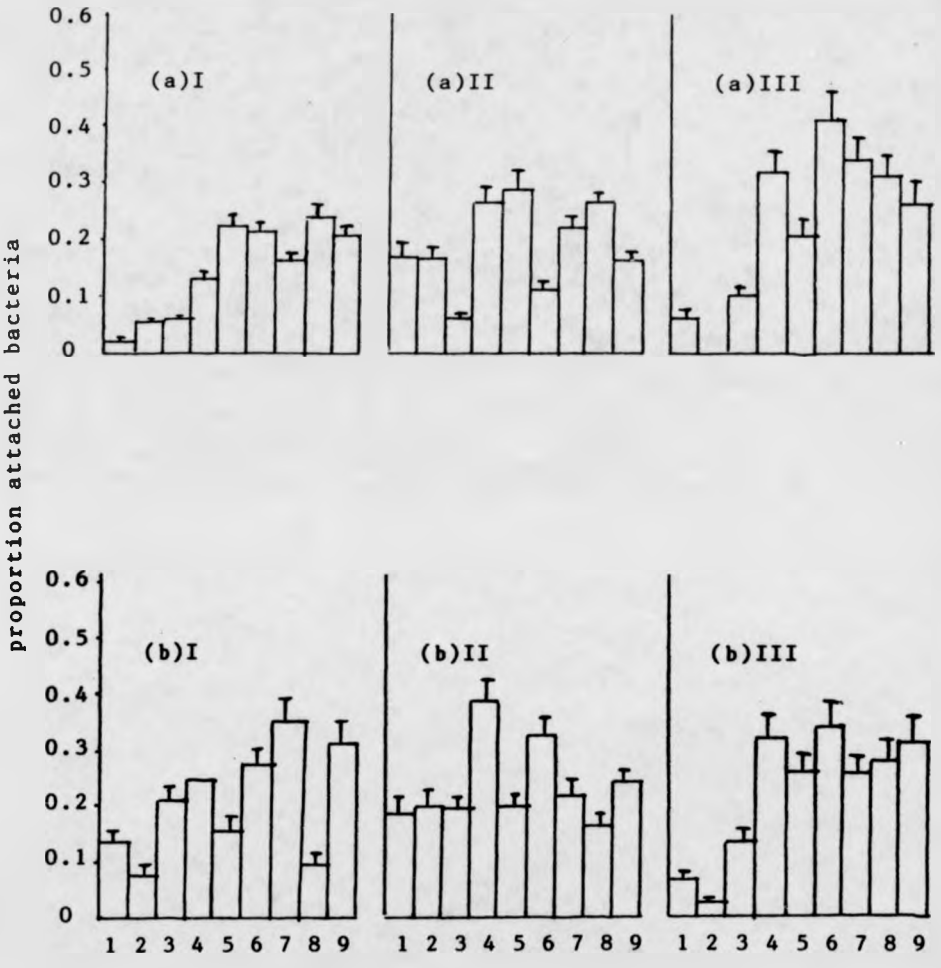

FIGURE 4.2
Attachment of (a) Aeromonas, (b) Chromobacterium in (I) glucose media, (II) mannose media and (III) sucrose media.

N.B.
(1) The media 1-9 and carbon sources are as indicated in the text. The medium and carbon source listed were the growth medium and the attachment medium.

(2) All cultures were growth in batch culture at 15°C. The temperature at which attachment was carried out was also 15°C.

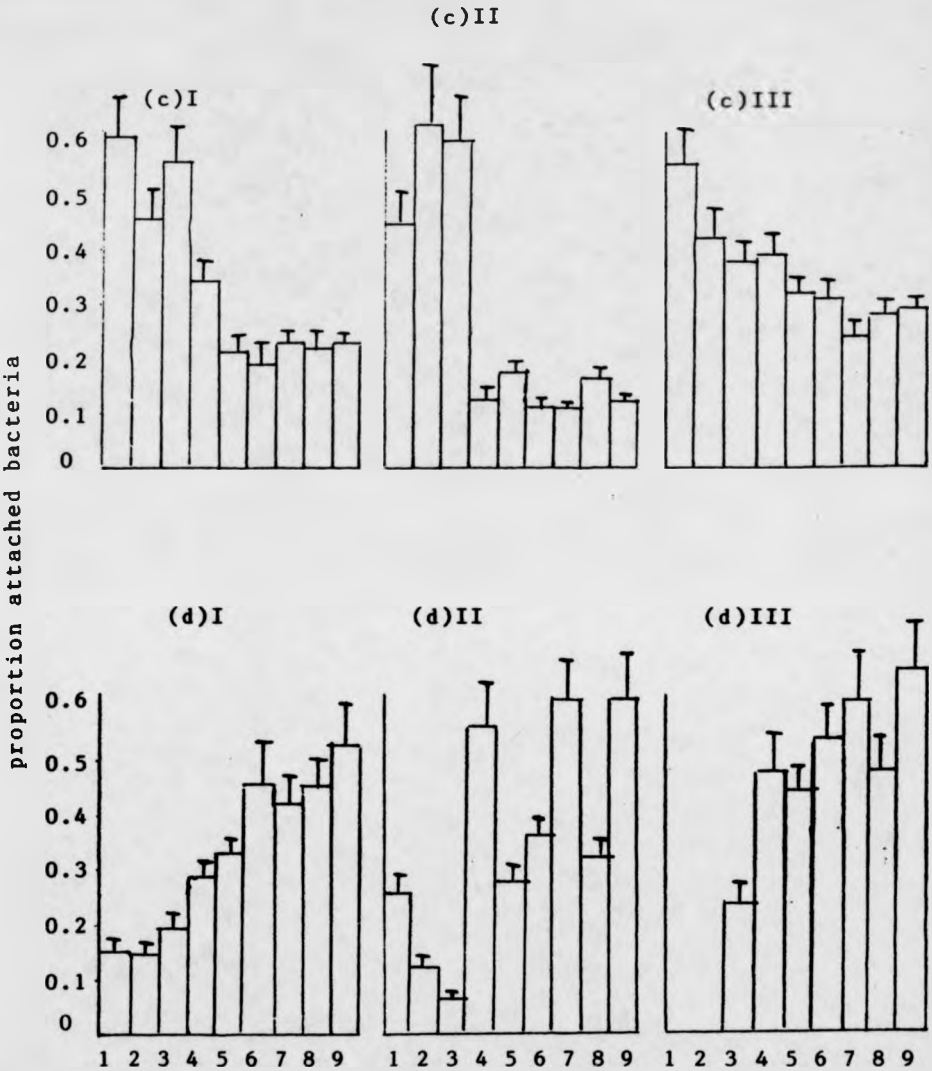

FIGURE 4.2 (Continued)
Attachment of (c) the coryneform, (d) Staphylococcus in (I) glucose media, (II) mannose media and (III) sucrose media.

N.B.
(1) The media 1-9 and carbon sources are as indicated in the text. The medium and carbon source listed were the growth medium and the attachment medium.

(2) All cultures were grown in batch culture at 15°C. The temperature at which attachment was carried out was also 15°C.

In general, Aeromonas attachment increased with increasing carbon concentration. This was most noticeable with the glucose medium (FIGURE 4.2 (a)I) but the same trend was apparent with the mannose (FIGURE 4.2 (a)II) and sucrose media (FIGURE 4.2 (a)III). In all cases, attachment seemed to reach a maximum at about 0.2 g carbon l^{-1}. Nitrogen concentration had little discernible effect on attachment of Aeromonas.

Chromobacterium attachment was studied in glucose Media (4) and (5). The attachment was significantly greater ($P>0.05$) in the glucose carbon-limited Medium (4) compared to the attachment in the glucose carbon-to-nitrogen limited Medium (5) (FIGURE 4.2 (b)I). This was the opposite of the result obtained for Aeromonas.

In general there was no correlation between attachment of Chromobacterium and carbon concentration although in the glucose and sucrose media, attachment was highest at the higher carbon concentrations (FIGURE 4.2 (b)I + (b)III). However, there was more correlation with nitrogen content, with Chromobacterium attachment being higher in every case where, although the carbon concentration remained constant, the nitrogen concentration was increased. This was most apparent with the glucose medium (FIGURE 4.2 (b)I). In the mannose and sucrose media there was a peak of attachment at about 0.38 g NH_4CL l^{-1} (FIGURE 4.2 (b)II + (b)III). The results obtained in these pure culture attachment experiments were similar to those obtained by McEldowney (1986).

Coryneform attachment decreased as carbon concentration increased (FIGURE 4.2 (c)I, (c)II, and (c)III). Attachment was always highest in the lowest carbon concentrations and in each case plateaued at a lower level at concentrations above 0.2 g carbon l^{-1}. Nitrogen concentration

seemed to have a similar but less dramatic effect on the attachment of the coryneform.

As with Aeromonas. Staphylococcus attachment increased as the carbon concentration increased for all three carbon sources (FIGURE 4.2 (d)I, (d)II and (d)III). In each case at constant carbon concentration, the increase in nitrogen concentration also increased attachment. This was not seen with Aeromonas.

Overall four different patterns of attachment were seen with the four species used. These can be summarised;

Aeromonas - Attachment increased with carbon concentration but nitrogen concentration had no effect.

Chromobacterium - No effect for carbon concentration but increases in nitrogen concentration increased attachment.

The coryneform - Attachment decreased with increased carbon concentration.

Staphylococcus - Attachment increased with carbon concentration and nitrogen concentration also had a positive effect on attachment.

4.4.2 Bacterial Attachment in Continuous Culture Grown Cells.

These changes in the attachment of bacteria with growth conditions were still apparent when the bacteria were grown in continuous culture, for example the coryneform attached to a greater extent in the glucose Medium (4) than in the mannose Medium (4) (FIGURE 4.3 (c)g and (c)m). The carbon-to-nitroge.. ratio could still influence attachment in continuous culture, for example Staphylococcus attached significantly better ($P>0.05$) in the glucose Medium (6) which was sufficient in carbon and nitrogen compared to attachment in Medium (5) which was nitrogen-

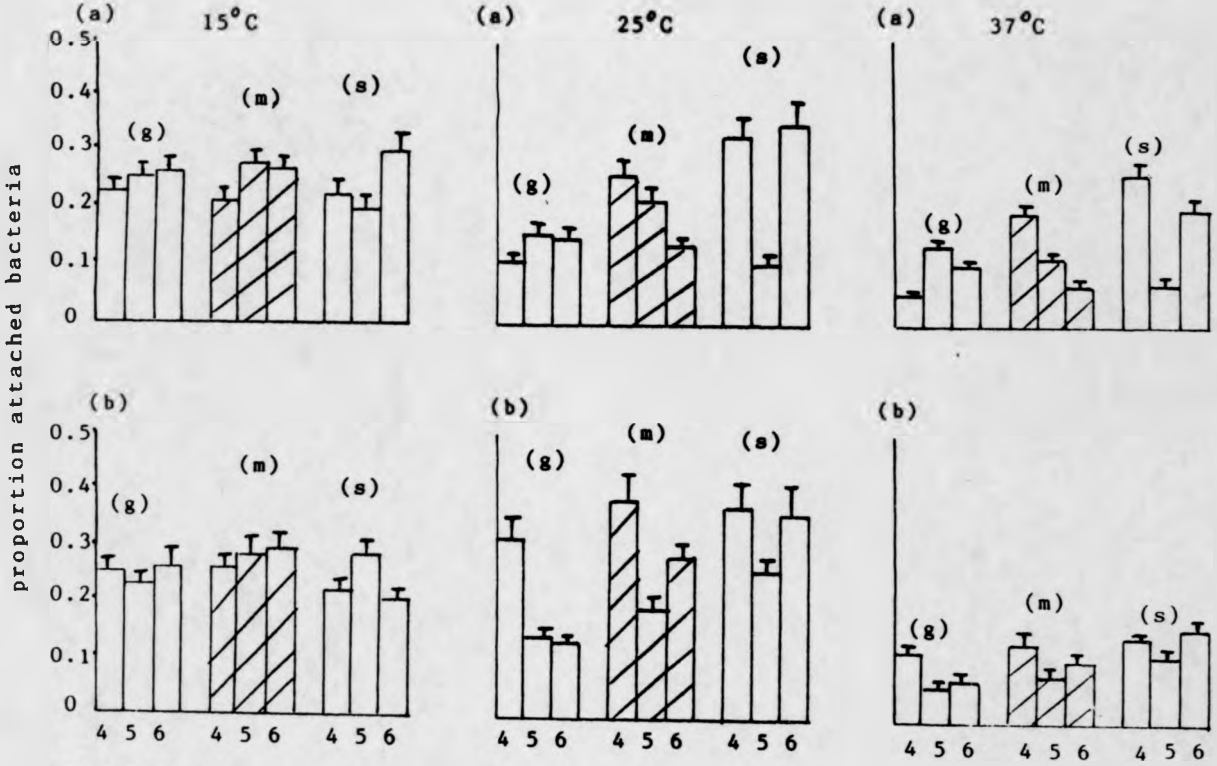

FIGURE 4.3
The attachment of (a) Aeromonas, (b) Chromobacterium in (g) glucose media, (m) mannose media and (s) sucrose media.

N.B. - The bacteria were grown in continuous culture at 15°C. The attachments were performed in batch at the temperatures indicated.

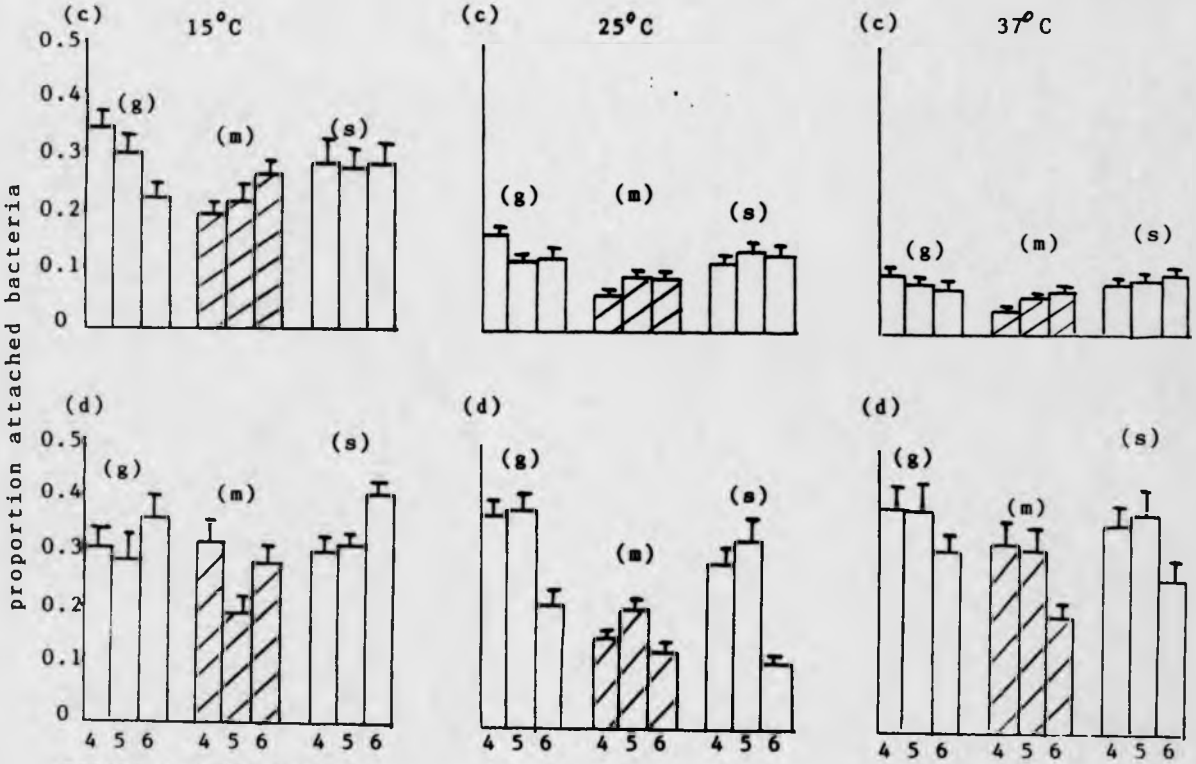

FIGURE 4.3 (Continued)
The attachment of (c) the coryneform, (d) Staphylococcus in (g) glucose media, (m) mannose media and (s) sucrose media.

N.B. - The bacteria were grown in continuous culture at 15°C.
The attachments were performed in batch at the temperatures indicated.

limited (FIGURE 4.3 (d)g). The results obtained however, were still dependent on the bacterium and the carbon source used in the attachment experiment.

In most cases it was observed that the bacteria grown in continuous culture (FIGURE 4.3 15°C) attached in similar numbers to the glass coverslips as the bacteria grown in batch culture (FIGURE 4.2). The only exception to this was the Staphylococcus which attached, in all but glucose Medium (5), to a lesser extent after continuous culture compared to batch culture grown cells (FIGURE 4.2 (d) and FIGURE 4.3 (d) 15°C). The response by a bacterium to different carbon sources when grown in batch or continuous culture could also be different. An example of this was seen when the coryneform was investigated after growth in continuous culture. Out of all the media used the coryneform was found to attach to a greater extent in glucose Media (4) and (5). When the coryneform was grown in batch culture in the different media, the greatest number of attached bacteria were observed in sucrose Media (4) and (5). Variations observed in the attachment of bacteria due to changes in the carbon-to-nitrogen ratio of the media varied depending on whether the bacteria were grown in batch or continuous culture. Bacteria grown in mannose Medium in batch culture usually attached in greater numbers in the carbon-limited Medium (4) (FIGURE 4.2 (a + b + c)II), compared to the continuous culture-grown cells which usually attached to a greater extent in the mannose carbon-to-nitrogen sufficient Medium 6 (FIGURE 4.3 (m) 15°C).

In general the conclusions reached regarding the attachment of the batch-grown cells in response to changing carbon and nitrogen ratios also apply here. The fact that the results obtained for the pure culture

bacteria in batch culture differed from the results when the bacteria were grown in continuous culture, again suggests that changes in the growth conditions of a bacterium can influence its attachment ability

4.4.3 Effect of Temperature on the Attachment of Batch Grown Bacteria.

When the four bacterial species were grown in batch culture in the Media 4, 5 and 6 with the three carbon sources glucose, mannose and sucrose, variations in bacterial attachment were observed when the attachment temperature was altered. Aeromonas demonstrated little change in its attachment ability between the temperatures of 15°C and 25°C (FIGURE 4.4 (a)). When the temperature was increased to 37°C in every case Aeromonas attachment increased compared to the results obtained at 15°C and 25°C. These results with an increase in bacterial attachment with an increase in temperature are similar to those reported by Fletcher (1977). These results, however, were influenced by the growth conditions as would be expected from the results in Section 4.4.1. An example of this can be seen when Aeromonas attachment in sucrose Media (5) and (6) is investigated at 15°C, 25°C and 37°C. Aeromonas attachment in sucrose Medium (6) changes very little as the temperature increased. In sucrose Medium (5) however, Aeromonas attachment increased significantly ($P>0.05$) as the temperature increased from 25°C to 37°C.

The results for the attachment of Chromobacterium at different temperatures were similar to the results obtained for Aeromonas (FIGURE 4.4 (b)). Chromobacterium demonstrated little change in attachment ability between temperatures of 15°C and 25°C. When the temperature was increased to 37°C however, there was an increase in the attachment of

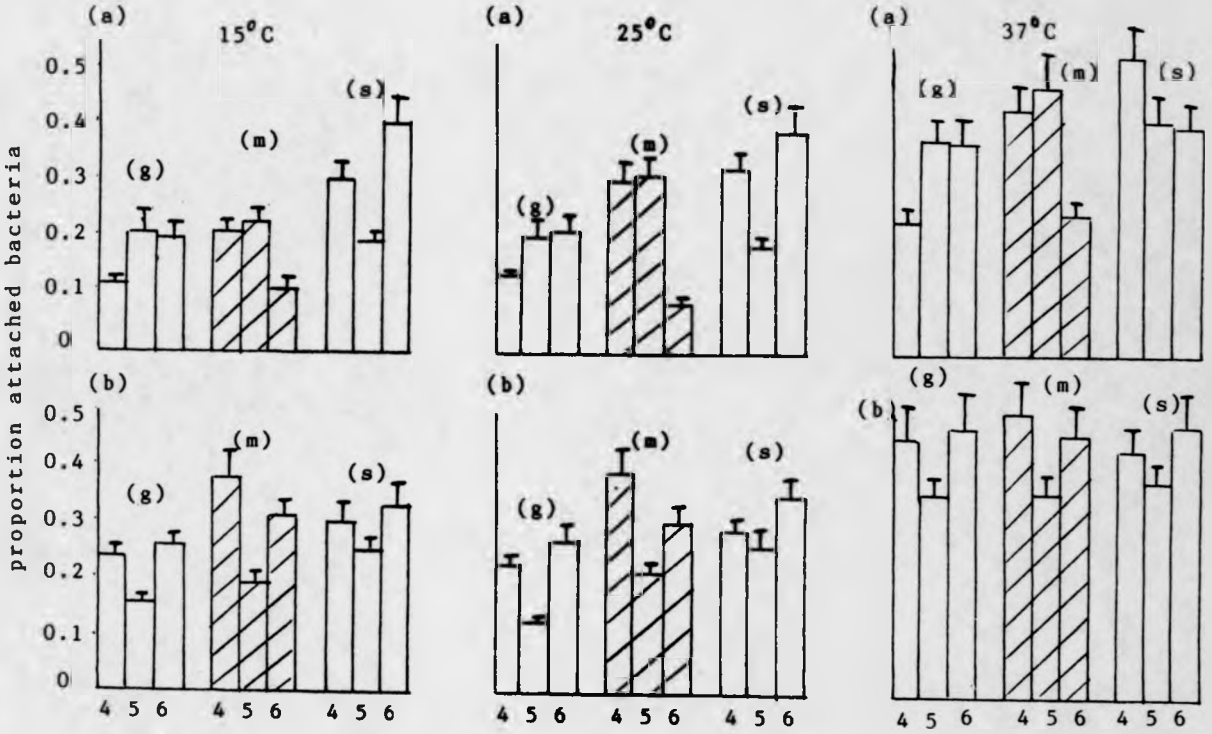

FIGURE 4.4
The attachment of (a) Aeromonas, (b) Chromobacterium in (g) glucose media, (m) mannose media and (s) sucrose media.

N.B. - The bacteria were grown in batch culture at 15°C.
The attachments were also performed in the growth medium in batch at the temperatures indicated.

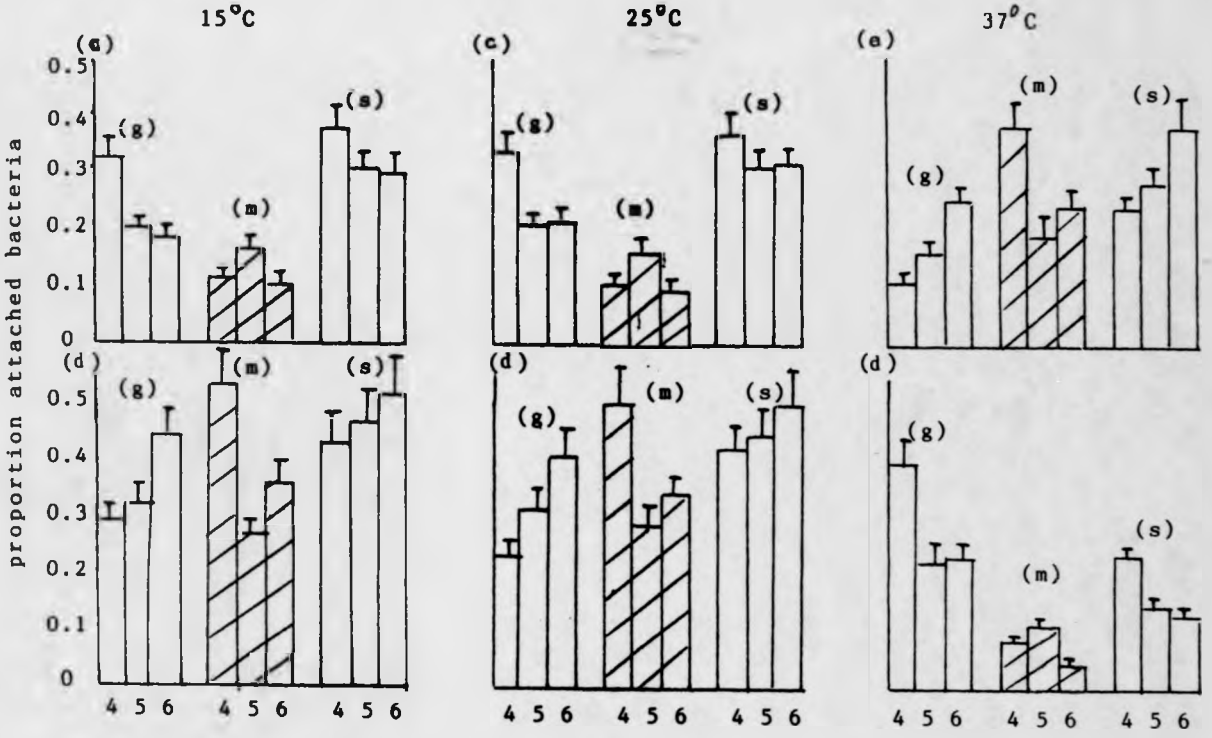

FIGURE 4.4 (Continued)
The attachment of (c) the coryneform, (d) Staphylococcus in (g) glucose media, (m) mannose media and (s) sucrose media.

N.B. - The bacteria were grown in batch culture at 15°C.
The attachments were also performed in the growth medium in batch at the temperatures indicated.

Chromobacterium in all cases compared to the results obtained at 15°C and 25°C.

Unlike with Aeromonas and Chromobacterium cultures, the response of the attachment of the coryneform to the glass surfaces at different temperatures was more complex (FIGURE 4.4 (c)). The coryneform attachment did not change when the attachment temperature was increased from 15°C to 25°C. When the temperature was increased to 37°C, marked and varied changes in the attachment of the coryneform were observed (FIGURE 4.4 (c)). In the mannose medium, the coryneform attachment increased as the temperature was increased from 25°C to 37°C, the extent of this increase was seen to vary with the carbon and nitrogen concentrations used and the carbon-to-nitrogen ratios. In glucose and sucrose media, the coryneform attachment also changed as the temperature was increased fron 25°C to 37°C, however, these changes in the coryneform attachment were influenced greatly by the different media used. These results suggest that the medium composition changed the cell surface in a more complex manner than this study examined, such that the effects of temperature on coryneform attachment seen here were artefactual and the real effects were due to changes in the cell surface engineered by changes in the growth conditions.

The results obtained for the attachment of Staphylococcus at different temperatures again demonstrated that there was little change in the attachment of Staphylococcus as the temperature was increased from 15°C to 25°C (FIGURE 4.4 (d)). The changes in attachment that did occur were again seen when the temperature was increased from 25°C to 37°C. These changes again varied with the medium composition, however, in all the media apart from glucose Medium (4) there was a significant

(P>0.05) decrease in the coryneform attachment as the temperature was increased from 25°C to 37°C.

4.4.4 The Effects of Temperature on the Attachment of Continuous Culture Grown Cells.

The results for the attachment of bacteria in batch, after growth in continuous culture at different temperatures, are shown in FIGURE 4.3. The results for Aeromonas and Chromobacterium demonstrated that the bacteria generally attached similarly at 15°C and 25°C although significant differences were sometimes seen in the attachment of the bacteria in different media at the different temperatures (FIGURE 4.3 (a)and (b)). An example of this can be seen when the attachment of Chromobacterium was investigated in glucose media at 15°C and 25°C. As the temperature was increased from 15°C to 25°C the attachment of Chromobacterium in glucose Medium (5) and (6) decreased significantly (P>0.05). In glucose Medium (4), however, the attachment of the Chromobacterium was seen to increase as the temperature was increased from 15°C to 25°C. These results were similar to the batch culture attachment experiment results (FIGURE 4.4 (a) and (b)).

When the temperature was increased to 37°C there was a decrease in Aeromonas and Chromobacterium attachment in all cases compared to the 25°C and 15°C. The extent of this attachment decrease, however, was still dependent on the bacterium investigated and the medium used. These results were the opposite to those obtained with batch culture cells, indicating that growth conditions can greatly influence bacterial attachment.

With the coryneform there was a significant decrease in attachment as the temperature increased from 15°C to 25°C and to a lesser extent

from 25°C to 37°C (FIGURE 4.3 (c)). These results were again different from those obtained with batch culture cells (FIGURE 4.4 (c)). In batch culture-grown cells medium composition was thought to greatly influence coryneform attachment. These results indicated that all growth conditions could be important in the attachment of coryneform.

When the attachment of Staphylococcus was investigated a complex pattern of attachment was observed (FIGURE 4.3 (d)). There was a minimum of attachment at 25°C in some cases (e.g mannose Media (4) and (5)), or an increase in attachment with temperature (e.g mannose Media (4) and (5)). This is comparable with the results seen with the bacteria grown in batch culture, with the growth conditions including media composition influencing the attachment of Staphylococcus at the different attachment temperatures.

4.5 DISCUSSION

4.5.1 Changes in the Bacterial Surface Characteristics with Nutrient Conditions.

The results for the attachment of bacteria in pure culture in media with different carbon source, carbon-to-nitrogen ratio, carbon concentration and growth rate are similar to those observed by McEldowney (1986). The culture conditions above were seen to influence the bacterial attachment to solid surfaces. Zvyagintsev (1959) demonstrated that the composition of the growth medium influenced the attachment of bacteria to surfaces. The importance of the growth medium was also demonstrated for a Vibrio species where attachment was influenced by the growth medium used (Jones, 1976). Often changes in a single component of the growth medium were enough to cause changes in the attachment of microorganisms to the surfaces used. Changing the

carbon source or carbon concentration of a medium has been shown to influence the attachment of microorganisms to surfaces (McCourtie, 1981, 1984, 1985; Rodger, 1990). Changing the growth medium can lead to morphological changes in the bacterium (Ensign, 1964). This could result in changes to the surface characteristics of a bacterium which could in turn result in changes in bacterial attachment.

A method for investigating differences in bacterial cell surfaces was introduced by Oss (1975) and has been used since by other researchers (Fletcher, 1982; McEldowney, 1986). This method involves the measurement of contact angles of the bacterial surface. When the contact angles of bacteria grown on a series of different agars were investigated, the contact angles were seen to vary with the agar used and the bacterial species (Baier, 1980). These results suggested a possible relationship between growth conditions, attachment and the bacterial surface characteristics. Evidence to support this claim can be seen when the cell wall structure of a bacterium is irreversibly altered by treatment with heat or formalin. These bacteria attach less readily to solid surfaces than the control (untreated) cells. The attachment of cells killed by ultra-violet light, on the other hand, which show little structural change to their surface characteristics is not affected to any great extent (Meadows, 1971: Fletcher, 1980).

The changes in attachment observed with the above treatments depend on the bacterial species present. When bacteria were treated with formaldehyde, a Micrococcus species increased in attachment, however, the attachment of a Streptococcus species did not change. The treatments with heat, formalin and formaldehyde, were thought to change the macromolecular structure of the bacterial surface. It was possible that

a new macromolecular arrangement at the bacterial surface could increase or decrease bacterial attachment to a solid surface, or even leave the attachment of the bacteria unaffected.

The cell wall constituents of bacteria (Chapter 1) can be influenced by numerous elements in the growth medium. The effects of factors such as carbon-to-nitrogen ratio of the medium and the carbon source of the medium, have been shown to be important in the attachment of bacteria to surfaces and can influence the cell wall constituents of bacteria.

Polymers on the surface of bacteria have been shown to be affected by the carbon-to-nitrogen ratio and carbon source of the medium in which the bacteria were grown. The production of a polysaccharide by a Pseudomonas species was observed under nitrogen-limitation conditions, but not glucose-limitation (Williams, 1978). During these studies, the production of polymer in the nitrogen-limited medium could be increased if phosphate and glucose were present in excess. The results obtained in carbon- and nitrogen-limited media vary depending on the bacterial species present. Jarmin (1978) demonstrated that an Azotobacter species produced high amounts of alginic acid under a range of limiting conditions. Variations were also demonstrated within bacterial groups with one Pseudomonas species producing large amounts of alginic acid only under carbon-limited conditions, while another Pseudomonas species produced this polysaccharide in large amounts under carbon- and nitrogen-limited conditions (Mion, 1978: Elwood, 1982). Pace (1980), demonstrated that under different growth limiting conditions, the quantities of rhamnose in the polysaccharide of a Xanthomonas species was seen to vary. These results suggest that the effects of the limiting

nutrient on attachment varied with the bacterial species, as was found in these studies.

The carbon source in the medium can also influence the polymer on the surface of the bacteria. This is probably more important in the case of homopolysaccharides, which need a specific carbon source for their formation. It is possible that the carbon source needed for the homopolysaccharide formation was present, however, it could not be utilised due to the specificty of the synthesising enzymes. The formation of heteropolysaccharides from utilisable carbon sources can vary in quantity depending on the carbon source present. Wilkinson (1958) demonstrated that polysaccharide production was less in high acetate-to-glucose medium compared to lower acetate-to-glucose medium. The polysaccharide produced by a Pseudomonas species did not vary with the carbon source but the quantity produced was seen to vary (Williams, 1977).

The production of polymer can also be affected by growth conditions. These results, however, are related to the bacterial species being investigated. For example, a polysaccharide produced by a Chromobacterium species increased when an amino acid was substituted for ammonia as a nitrogen source (Corpe, 1964). As well as the composition of polymer varying with nutrient conditions, its physical relationship with the bacterial cell wall can also change. This was seen with a Chromobacterium species which was grown in the presence of tryptone. The bacterium did not produce a polymer matrix but extruded polymer into the medium.

The results for the continuous culture attachment experiments indicate that the growth conditions such as carbon source affect

attachment in similar way. The dilution rates used in these experiments could also affect the polysaccharide quantity produced by bacteria. The quantity of polymer produced again varies with the bactreria being studied. This was demonstrated by Rudd (1982), when K. aerogenes polymer production increased as the dilution rate decreased. This was not the case, however, when a Pseudomonas species was studied, as the same amount of alginic acid was produced at all growth rates.

The physical form of the polymer produced by bacteria can also be affected by the growth rate. K. aerogenes produces most of its polymer in a colloidal form at low dilution rates and this changes to most of the polymer being in a soluble form at higher dilution rates (Rudd, 1982). These differences can reflect changes in the structure of the polymer or in a change in the interaction between the polymer and the cell wall.

Differences in polymer production have been shown in bacteria grown in batch and continuous culture. K. aerogenes polymer production was greater in continuous culture than in batch culture (Rudd, 1982). In our experiments, bacteria were grown in continuous culture for 3 days. This could have allowed them to produce more polymer on their surfaces compared to the cells grown in batch culture. This might explain the differences observed in bacterial attachment between batch-grown and continuous-culture grown bacteria.

4.5.2 Nutrient Source and Effects on Developing Biofilms

When the influence of nutrient conditions on the attachment of bacteria are investigated, it is possible that the nutrients present could influence bacterial attachment without changing the bacterial

surface directly. The attachment rate of a Pseudomonas species did not change when the glucose concentration was between 100-1000 mg l^{-1}. (Lawrence, 1987a). Other researchers, however, have reported glucose inhibiting Pseudomonas attachment in concentrations in excess of 14 mg l^{-1} (Marshall, 1976). The different effects of glucose on the attachment of Pseudomonas could be explained by different experimental procedures being employed, as was discussed in Chapter 3 or by different species of Pseudomonas attaching differently in different media, as was seen in Table 3.1. Caldwell (1986) has demonstrated that the growth of bacteria on surfaces is flow-dependent at low carbon concentrations and flow-independent at high carbon concentrations. It was suggested that in the absence of laminar flow velocities, the surface could be substrate depleted and this would affect bacterial attachment. This would be unlikely to be important in these laboratory studies as the bacterial attachment was performed in batch, however, this could be important under other growth conditions.

The different nutrients present in the aquatic environment could also influence the bacterial biofilm that developed. When a carbon source enters an aquatic system, the rate at which it is utilised, was found to be influenced by the previous dissolved organic carbon compound and its concentration. The organisms present would therefore, be adapted to utilising the nutrients already in the system, and if a new nutrient was introduced into the system the organism best suited to utilising this nutrient would eventually dominate (Tranvik, 1987). This could lead to a new population developing which would result in new biofilms developing on the surfaces in this system. This could be of importance in the model system attachment experiments, Chapter 7.

Different nutrients also cause bacteria to react differently metabolically. Amino acids have been found to be taken up more rapidly by attached bacteria and glucose more rapidly by free-living bacteria (Fletcher, 1982). A substrate-specific response was noted here with amino acids producing more active bacteria and glucose leading to increased activity per bacterial cell. When considering attached bacteria, this could account for different biofilms developing in different media with more or less bacteria being present on the surface, depending on the nutrient used.

Many molecules could be used by bacteria as potential nutrient sources. In such cases these molecules could influence bacterial attachment to surfaces. Meadows, (1971) demonstrated that the attachment of E. coli and Pseudomonas was affected by proteins in the growth medium. Casein, for example, promoted the attachment of both these bacteria to the solid surfaces. Proteins, however, can cause bacteria to attach differently when in the growth media. In Meadows' experiments, gelatin was found to increase the attachment of the Pseudomonas to the solid surface, but not to affect the attachment of the E. coli to the surface. Bacteria are known to vary in the uptake methods used for proteins (Geesey, 1981). It could be that the way a protein influences bacterial attachment could be related to the uptake mechanism or lack of uptake mechanism in the bacteria being investigated. Proteins could also be involved in conditioning films which are known to influence bacterial attachment (Chapter 1).

The attachment of bacteria to solid surfaces can also be influenced by alcohols (Fletcher, 1983). If bacteria are attached in the presence of alcohols changes in bacterial attachment occurs. When bacteria are

pre-incubated with alcohols changes in bacterial attachment again occur. These changes in bacterial attachment were thought to be related to bacterial respiration rates.

The effects of different nutrient conditions on biofilms will depend on the biofilm being investigated. Burkholder (1990) demonstrated that microorganisms present in the hidden layers of a biofilm were relatively isolated from the water column nutrient source. It was suggested that the loosely attached microorganisms took up the bulk of the nutrients. These results suggest that the attachment of loosely attached bacteria would be more likely to be influenced by changing nutrient conditions than bacteria which are already part of the biofilm.

5.5.3 Temperature and Attachment.

When pure cultures of bacteria were used in attachment experiments, they varied in their attachment abilities between 15°C and 37°C. Changes in bacterial attachment due to temperature were also linked to changes in the media composition as was expected from the later discussions. The importance of temperature to bacterial attachment was indicated by experiments performed by Pedros (1983), Pederson (1982) and Rodger (1990). In these experiments, bacterial attachment to surfaces in the natural aquatic environment varied in the winter and summer months. The reason for this was thought to be related to changes in the water temperature. Variation in bacterial attachment has been reported by numerous researchers. Fletcher (1977) demonstrated that a Pseudomonas species increased its adhesion to a solid surface when the temperature was increased. Streptococcus faecium also increased its attachment when the temperature of the medium was increased, with attachment reaching an optimum, and then the numbers of attached bacteria fell as the

temperature was increased further. The results here indicate a maximum temperature range for each bacterium investigated as has been found elsewhere (Orstavik, 1977).

When a range of bacterial species and strains were investigated they were found to vary in their attachment to surfaces at different temperatures. It was suggested that different attachment mechanisms were being employed by these bacteria and this would account for the different attachment values obtained for the attachment of pure cultures of bacteria at different temperatures (Minato, 1976). The changes in attachment due to media composition are again thought to be related to changes in the bacterial surface structure. This was discussed in detail in Sections 4.5.1 and 4.5.2.

Further explanations for the effects of temperature on bacterial attachment are complicated, and involve changes in bacterial physiology, and also changes in the physico-chemical attachment interactions. Temperature can affect bacterial physiology as it can influence the rates of enzyme-controlled reaction rates. Factors such as cellular metabolism will vary with temperature. As the temperature increases, the cellular metabolism will increase as the temperature increases up to 40°C, as the optimum temperature for respiratory chain enzymes is around 40°C regardless of the bacterial species.

Physico-chemical parameters of adhesion are also important when considering the effects of temperature on attachment. Brownian motion is related to temperature and if bacteria are regarded as colloidal size particles then the bacteria will be in kinetic equilibrium with the molecules of the surrounding medium. This will mean that temperature changes in the surrounding medium will lead to changes in the Brownian

motion exhibited by the bacteria (Marshall, 1976). When the temperature of the surrounding medium is increased, there is a decrease in the medium viscosity which will lead to an increase in Brownian motion. This will lead to an increase in the probability of bacteria coming into contact with the solid substratum due to the increase in the random motion of the bacteria. In our experiments, in batch culture, the response of the coryneform attachment to increases in temperature does not suggest Brownian motion is involved, as the coryneform attachment, in some cases decreases as the temperature increases. This indicates that there must be other factors which are involved when temperature influences attachment. This has already been suggested as media compostion influences the attahcment of bacteria at different temperatures. These results suggest that the effects of temperature on attachment do not involve Brownian motion, however, this could be important under other unknown circumstances. During our experiments the attachment of Aeromonas and Chromobacterium decreased as the temperature was decreased from 37°C to 25°C. This would fit in with the Brownian motion theory as the viscosity of the medium would increase as the temperature decreased so the bacteria would not come into contact with the surface as frequently. In this case, Brownian motion could be seen as influencing the bacterial attachment to solid surfaces.

If the bacteria use an adhesive to attach to the surfaces the viscosity of the adhesive could change with temperature and this would affect the attachment of the bacteria to surfaces. Temperature could also influence the balance between physical and chemical adsorption. This system is very complex and depends on whether the adsorption process is endothermic or exothermic (Kipling, 1965).

Research has shown that low temperatures are best for physical adsorption while higher temperatures favour chemiosorption. Physical adsorption increases at higher temperatures due to hydrophobic bonding and other non-specific attractive forces such as London-van der Waals forces or electrostatic bonding (Shaw, 1970; Ben-Nain, 1980). Chemiosorption is a stronger more selective interaction and it is doubtful if these interactions are involved in bacterial attachment. This indicates that physical adsorption is most relevant when considering bacterial attachment.

Physical adsorption will again involve the cell surface, and changing the temperature of the surrounding liquid phase could alter the cell surface configuration and this could increase or decrease the attachment to a surface. Other considerations must also be taken into account as the effect of temperature on attachment cannot always be explained by these parameters. A good example of this is that if hydrophobic interactions are favoured by a increase in temperature, then it would be expected that a Flexibacterium species with hydrophobic poles would increase its attachment to a surface with an increase in temperature. This is not the case so other factors must be involved in the attachment of the bacterium to the surface (Ben-Nain, 1980).

The importance of polymeric material in the attachment of bacteria to solid surfaces has already been discussed (Chapter 1). Fyfe (1986) demonstrated that temperature could affect the amount of extracellular material produced by bacteria. Fyfe (1987) also demonstrated that the temperature of water could influence the extracellular compounds produced by bacteria. Polymers have also been shown to decrease in their adhesive strengh with an increase in temperature. This is due to changes

in the viscosity of the polymers (Stanley, 1967). This will be of most importance in bacteria which use polymeric material for attachment. Temperature, however, is not always detrimental to bacterial extracellular proteins. Kang (1981) demonstrated that polysaccharides could withstand high changes in temperature. Temperature has also been shown not to influence the production of polymer by bacteria (Fletcher, 1972). These results do indicate the effects of temperature on bacterial extracellular material is complicated.

CHAPTER FIVE

EFFECT OF pH AND ELECTROLYTE TYPE AND CONCENTRATION ON THE ATTACHMENT OF BACTERIA.

5.1 AIM

To study the effect of varying the pH and the electrolyte concentration in the attachment medium on the attachment of bacteria.

5.2 INTRODUCTION

pH has been shown to influence the attachment of pure cultures of bacteria to surfaces. Bacteria have been reported to attach more readily at low pH (Stanley, 1967; Hwa 1984), but different bacterial species demonstrated a different pH optima for attachment. In this study, the influence of pH on the attachment of pure cultures of bacteria was investigated.

As discussed in Chapter 4 bacterial attachment is influenced greatly by physico-chemical phenomena. Harden (1953) demonstrated that, at normal physiological pH's, bacteria have a predominantly negative charge. Therefore, the net effect of positively charged surfaces coming into contact with the bacterial surface would be attractive and negatively charged surfaces would be involved in repulsive interactions. Due to this phenomenon, bacterial cells tend to attract cations and repel anions. It is therefore cations which form a diffuse layer near the surface called the diffuse electrical double-layer. The electrolyte concentration of the surrounding medium and the valency of the electrolyte used can influence the thickness of the diffuse electrical double-layer. If the electrolyte concentration and/or electrolyte valency is increased the diffuse electrical double-layer becomes more compact with the net result of being the thickness of the double-layer

decreases. The diffuse electrical double-layer thickness (1/K) can be calculated for symmetrical electrolytes in aqueous solutions as:

$$K = 0.327 . Zc^{\frac{1}{2}} . 10^{8} .$$

where Z is the valency and c the molar concentration of the electrolyte (Shaw, 1970).

According to the DLVO theory, the potential energy of interaction between two approaching particles (bacterium and the surface) is estimated by the electrical repulsion energies in their overlapping electrical double-layers and by the attraction energies of van der Waals forces. This results in the balance between two forces varying with the concentration and valency of the electrolyte present, and the interparticulate distance between the particles. Although Van der Waals forces are unaltered by changes, in the type or concentration of electrolyte repulsion energies between particles vary with the electrical double-layer. Low electrolyte concentrations and/or low electrolyte valency result in large 1/K values which do not promote particle interaction. FIGURE 5.1 demonstrates how at high 1/K there is a net attraction close to the surface, the primary minimum. The repulsion energy at a greater distance from the surface here is very large and maybe to large for the bacteria approaching the surface to overcome. This would not promote particle interaction and therefore not promote bacterial attachment to the surface.

At high electrolyte concentrations and/or high electrolyte valency however the 1/K value is lower and promotes particle interaction. At small 1/K another point of attraction develops the secondary miminum (FIGURE 5.2). As discussed in the introduction the reversible stage of bacterial attachment is thought to occur at the secondary minimum

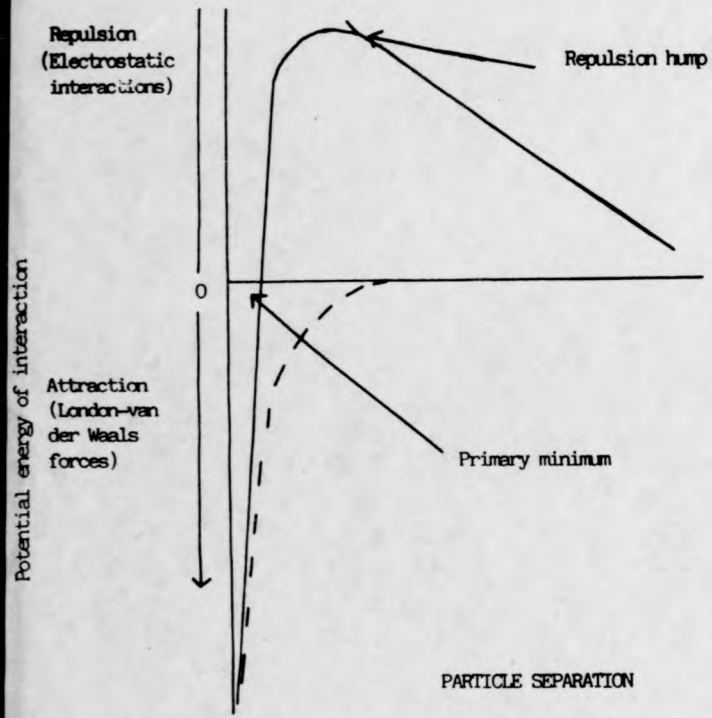

Figure 5.1 Potential energy of interaction between two particles at large 1/K. The dotted lines represent the attractive and repulsive energies, the solid line represents the resultant energy of interaction

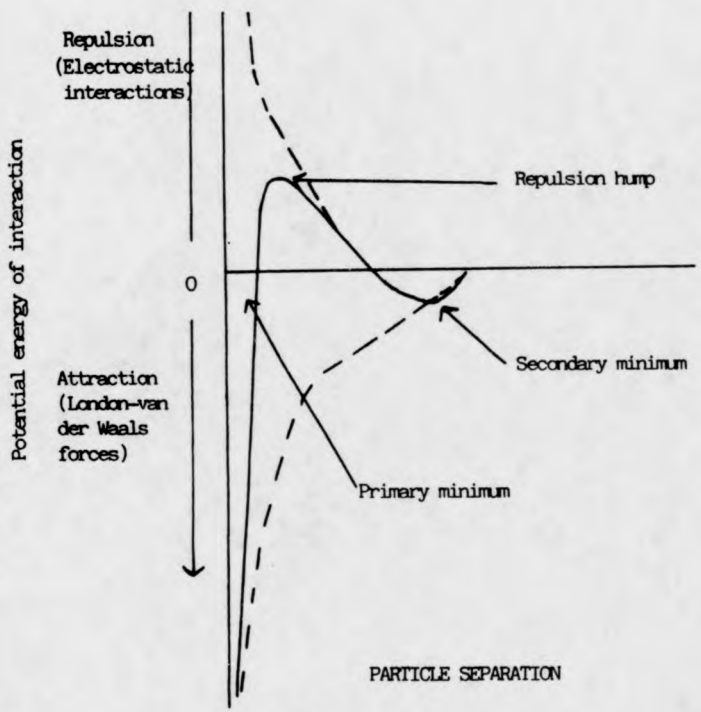

Figure 5.2 Potential energy of interaction between two particles at small 1/K. The dotted lines represent the attractive and repulsive energies, the solid line represents the resultant energy of interaction

(Marshall, 1971). Therefore, as the values of 1/K can influence the primary and secondary minima, 1/K could influence the attachment of bacteria to surfaces. This theory allows us to investigate how the attachment of pure culture bacteria varies with different 1/K values.

5.3 MATERIALS AND METHODS

5.3.1 Bacteria and Inoculation

The bacterial species studied in these attachment experiments were Aeromonas, Staphylococcus and the coryneform. These bacteria were chosen as they had demonstrated different attachment abilities in the previous attachment experiments (Chapter 4). The experimental procedure outlined in Section 3.3.1 was followed to prepare stock cultures of these bacteria. 1ml of each stock culture was used to inoculate the 100ml batch cultures used to grow the bacterial cells required.

5.3.2 pH and Attachment

The importance of pH on attachment was studied after the bacteria had been grown in batch culture in glucose Medium (6) at 15°C as described in Section 4.3.2. Aeromonas, coryneform and Staphylococcus used in these experiments were grown in glucose Medium (6) until late stationary phase. The bacteria were harvested by centrifugation at 11,000 av.g for 15 mins. The bacterial cells were washed twice in 0.01M Hepes buffer (pH 7.4) and resuspended in 100ml of a mixed buffer system adjusted to pH's ranging from pH 4.0 to pH 9.0, to an optical density of 0.1 at 540 nm.

The mixed buffer system consisted of 0.9 g 3,3-dimethylglutaric acid puriss (KOCH-Light Laboratories Ltd, Bucks), 1.95 g MES (2(N-morpholino)ethanesulfonic acid), 1.9 g ADA (N-(2-Acetamido)-Iminodiacetic acid), 2.38 g HEPES (N-2-Hydroxyethyl-piperazine-N-2-

ethanesulfonic acid) and 1.58 g TRIZMA HCL (Tris(hydroxymethyl aminomethane hydrochloride) (all Sigma , Poole) in 900 ml of distilled water. The pH of the buffer was then adjusted to the required pH with 1M HCL or 1M NaOH, and the volume adjusted to 1000 ml (May and Baker Ltd, London).

Once the bacterial cells had been resuspended, they were incubated in a shaking incubator at 150 rpm at 15°C for 10 mins, to stabilise them to their new conditions. The glass surfaces were aseptically added to the flasks and the attachment experiments were performed as in Section 4.3.2, the only exception being that the glass surfaces were washed after attachment with sterile mixed buffer system at the attachment pH instead of the phosphate buffer used in these experiments.

5.3.3 Electrolyte Type and Concentration and Attachment.

In these experiments the same bacterial species described in Section 5.3.2 were used. The bacteria were grown in Medium (6) in batch culture. The 100 ml flasks were incubated in a shaking incubator at 15°C. Once late stationary phase was reached, the cells were harvested and washed as in Section 5.3.2. The bacteria were resuspended to an optical density of 0.1 at 540 nm in a series of electrolyte solutions in 0.01M Hepes at pH 7.4. The electrolyte concentrations used were 0M (control), 0.01M and 0.1M NaCl, 0.01M and 0.1M $MgCl_2$ and 0.1M $AlCl_3$.

The resuspended bacteria were incubated in a shaking incubator at 150 rpm at 15°C for 10 mins to stabilise the bacteria to the new conditions. Glass surfaces were aseptically added to the flasks and the attachment experiments were performed as in Section 5.3.2, the only exception being that the surfaces were washed in 0.01M Hepes pH 7.4, instead of the phosphate buffer after attachment.

5.4 RESULTS

5.4.1 The Effects of pH on the Attachment of Bacteria

The bacteria used in the pH attachment experiments were Aeromonas 2, the coryneform and Staphylococcus. The results for the attachment of these bacteria over a range of pH's can be seen in FIGURE 5.3. Aeromonas showed optimum attachment between pH 7.5 and pH 8.0. The coryneform showed optimum attachment at pH 6.5. Staphylococcus showed optimum attachment at pH 6.0.

These results demonstrate that different species have different reactions to pH variations, with attachment being optimum at different pH values. The coryneform was able to attach over the whole pH range, but Staphylococcus could not attach below pH 4.5 or above pH 8.5 and Aeromonas could not attach below pH 5.0. This again demonstrates that different species react differently over a pH range. The results obtained by Daniel (1980) suggested that the optimum pH for the attachment of bacteria was between pH 3 and pH 6. The results here do not agree with these findings with the best pH for attachment being between pH 6 and pH 8.

5.4.2 Electrical Double-Layer Thickness (1/K) and Attachment

The electrical double-layer thickness (1/K) was calculated using the equation in Section 5.2.1. The effect of 1/K on attachment of the three bacterial species Aeromonas 2, the coryneform and Staphylococcus is shown in FIGURE 5.4. Compared to the control, Aeromonas attached significantly ($P>0.05$) more when the 1/K was 0.3. This was expected due to the 1/K being small due to the high electrolyte concentration and high valency. When 1/K was 1.5, the attachment of Aeromonas was significantly ($P>0.05$) lower than the control value. The other 1/K

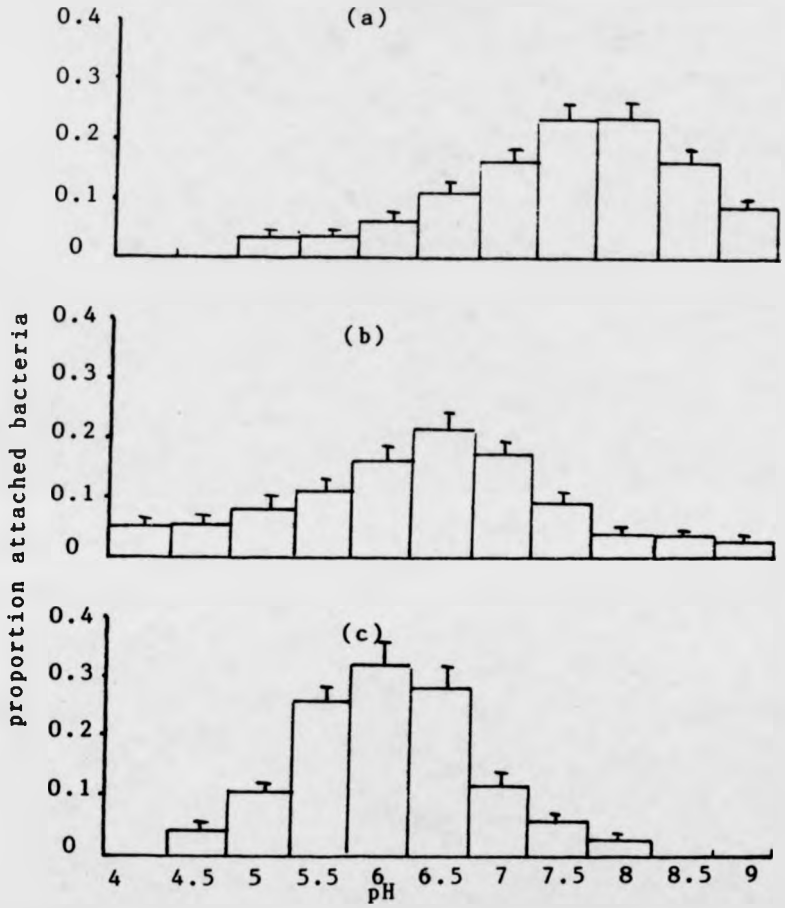

FIGURE 5.3
The attachment of (a) Aeromonas, (b) the coryneform and (c) Staphylococcus in a mixed buffer system.

N.B. - The buffer system was adjusted to a pH range of 4.0 to 9.0. The attachments were performed in this buffer system in a shaking incubator at 15°C.

The bacteria were first grown in batch in Medium 6 at 15°C, harvested and resuspended in the above buffer system.

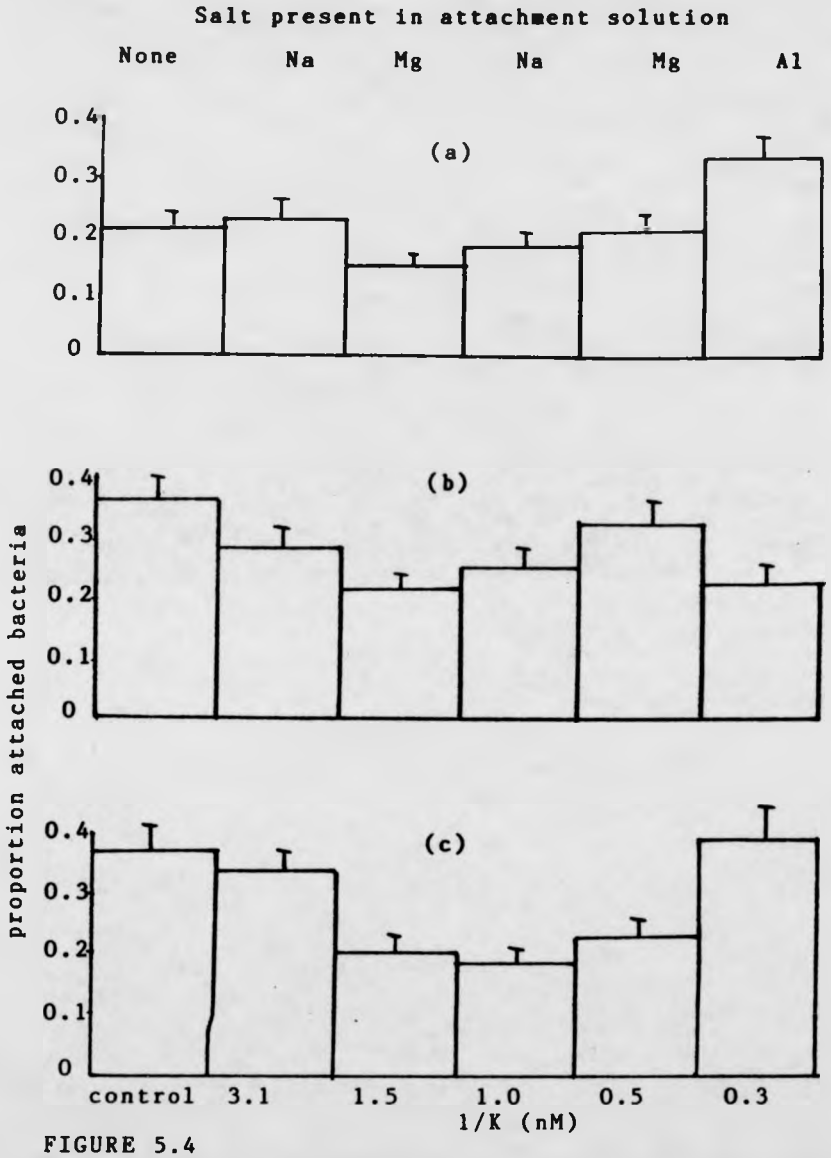

FIGURE 5.4
The effect of the electrical double-layer thickness on the attachment of (a) Aeromonas, (b) the coryneform and (c) Staphylococcus.

N.B.- The bacteria were attached in solutions with different electrolyte types and concentrations. These solutions were incubated in a shaking incubator at 15°C.

The bacteria were first grown in batch culture in Medium 6 at 15°C, harvested and resuspended in the solutions containing electrolytes.

values when compared to the control did not appear to influence Aeromonas attachment (FIGURE 5.4 (a)). These results were not expected as the different 1/K values were thought to influence the attachment of Aeromonas. FIGURE 5.4 (b) shows the effect of 1/K on the attachment of the coryneform. Compared to the control, the attachment of the coryneform is significantly ($P>0.05$) decreased at all 1/K values. These results were not those expected with changing 1/K values. The results for the attachment of Staphylococcus are shown in FIGURE 5.4 (c). Compared to the control value, the attachment of Staphylococcus did not vary significantly when the 1/K value was 3.1 or 0.3 but there was a significant difference between these values.

The results for the attachment of these bacteria at different 1/K values suggest that 1/K could influence bacterial attachment as in the case of Aeromonas at 1/K value 0.3. In most cases, however, the results were hard to interpret and suggested other factors were also influencing bacterial attachment in these experiments. The results in Chapter 4 have shown that different attachment media can influence bacterial attachment. In these experiments different salts were present in the attachment solutions and it is possible that these different salts influence bacterial attachment.

When the Al salt was present in the attachment solution both Aeromonas and Staphylococcus attached better in this solution than in the control solution. However, this is also the solution with the lowest 1/K value which was expected to provide the best attachment results. The attachment results with the Mg salt present suggested that either the Mg salt or the 1/K was decreasing the attachment of the bacteria in most cases. The attachment solutions with the Mg salt present showed that in

each case attachment was greater when the 1/K value was at its lowest, which agrees with Shaw's (1970) ideas. The results when a Na salt was present in the attachment media were the opposite of those obtained with the Mg salt. In each case the attachment of bacteria was greater in the solution with the greater 1/K. These results suggest that the attachment of bacteria in the presence of different electrolyte types at different concentrations is too complex for these experiments to help us understand fully what is happening.

5.5 DISCUSSION

5.5.1 pH and Attachment

The attachment of bacteria has been shown to be influenced by pH by numerous researchers. Daniels (1980) demonstrated that bacteria attached strongest to a variety of surfaces in the range pH 3 to pH 6. The attachment of bacteria to starch was found to vary over a range of pH's. Maximum attachment was found between pH 6 to pH 7, with attachment declining up to pH 10 when only 4% of the bacterial cells were attaching (Minato, 1976). In contrast to the above studies, Strenstrom (1989) demonstrated that the alteration of pH between 4 and 9 did not influence the attachment of a Salmonell species. pH has been found to influence the solid substratum to which the bacteria attach. The potential of a solid surface to take part in attachment reactions can change due to alterations in the surface related to pH. pH will lead to dissociation and therefore changes in the double layer of the surfaces. These effects will change with the surface being considered and will influence physical and chemical interactions.

If the pH at the surface differs from the pH of the liquid phase, it is possible that the adsorption of H^+ would occur at the interface and

this would result in changes in the surface charge. These changes in surface charge could influence bacterial physiological processes with pH optima (Hattori, 1961). Researchers have shown that the growth of bacteria is possible over a range of pH (McEldowney, 1976). This could indicate that physiological activity had little effect on attachment. During growth at the different pH's, it is possible that changes in the bacteria themselves occured. Changes in the surface of a bacterium due to pH could possibly influence the attachment of that bacterium.

Changes in the bacterial cell surface characteristics with pH have been well demonstrated (Plummer, 1961: Marshall, 1967; Marshall, 1973). The electrophoretic mobility of bacteria has been shown to change with pH. This is thought to be due to different proportions of ionogenic groups on the bacterial surface and their different dissociation points (Marshall, 1967).

Ionization of carboxyl groups which occur at alkaline pH's will confer a negative charge onto the bacterial cell and the ionization of amino acids at acidic conditions will confer a positive charge onto the bacterial cell. These suggest that the charge on the bacterial surface could change at different pH's. The proportions of carboxyl groups and amino acids on the bacterial surface can alter with the growth rate and growth conditions as was discussed in Chapter 4. It is not unexpected that changes in these groups result in changes in bacterial attachment. The lower the pH the more positive groups have been found on the surface, as the pH rises the number of groups with negative charges increases. Burns (1979) has suggested that the isoelectric point for most bacteria is pH 2 to pH 3, below which bacteria will be positively charged.

These systems don't often work in practice. This was demonstrated by Fletcher (1976) who used polystyrene surfaces which are mostly negatively charged and bacteria would be expected to attach in greater numbers at lower pH's where the majority of the base groups would be ionised, however, this was not the case. Changes in the surface characteristics caused by changes in pH could affect other phenomena such as the electric double layer thickness which could influence attachment directly. The pH of the liquid phase could also change the three-dimensional configuration of the bacterial surface molecules. This would occur if the ionisation of groups within the molecule was changed. If groups available for attachment were altered, this could result in an increase or decrease in bacterial attachment, depending on the balance of positively and negatively charged groups on the solid surface. Fontana (1979) found that the morphology of a Klebsiella species changed from cocci to bacilli at different pH's. Such changes in morphology could influence attachment.

pH has been reported to influence the bacterial surface in other ways. Different pH values can prevent the formation of primary polymer on a bacterial surface. If this polymer is important for bacterial attachment, the attachment of the bacterium concerned must be affected (Bush, 1968). The growth of bacteria on the walls of continuous culture vessels was shown to be due to polymer produced by these bacteria. pH was found to influence this wall growth and this was thought to be done by preventing the polymer concerned being produced or influencing the bacteria in some other way (Wilkinson, 1974; Atkinson, 1974). pH has also been shown to influence other attachment mechanisms of bacteria other than polymer. Corynebacterium renale attaches due to pili and the

attachment of this bacterium was found to be pH dependent. These studies indicate that pH can influence bacterial attachment in numerous ways.

5.5.2 Electric Double-Layer Thickness and Attachment

Marshall (1971) demonstrated that the attachment of a Pseudomonas to a glass surface did not take place if the 1/K value was 20 nm. At this value there was total repulsion between the Pseudomonas and the surface. As the 1/K value decreased the Pseudomonas was seen to increase in its reversible attachment levels to the surface. Therefore, the Pseudomonas was thought to be held at the secondary minimum (FIGURE 5.2). The importance of 1/K on the irreversible attachment of the Pseudomonas was unclear, the kinetic energy produced by the motility of the bacterium was insufficient to overcome the repulsion barrier between the primary and secondary minimum. The results obtained in these studies suggest that 1/K is not the prime factor involved in the permanent attachment of bacteria to surfaces. The 1/K was seen to increase or decrease bacterial attachment, depending on the electrolyte type and concentration also depending on the bacterial species present. Indeed, this was suggested by Vanhaecke (1990) who suggested that the influence of electrolytes on the attachment of bacteria to surfaces was dependent on the bacterial species being investigated.

Gingell (1978), demonstrated how cells attached better at high electrolyte concentrations (low 1/K). He suggested that the high electrolyte concentration enabled the bacteria to make contact with the primary minimum. This may have been the case in these experiments but electrolytes could influence bacterial attachment to surfaces in other ways. Rutter and Abbott (1978) demonstrated that the solid surface used in the attachment experiments could also influence the results obtained.

They demonstrated that the attachment of a Streptococcus increased to a rotary disc at ionic strenghs 0.03 to 0.1M, above this a reduction in Streptococcus attachment was observed. If a polystyrene surface was used, a reduction in bacterial attachment was observed when the electrolyte concentration was above 0.05M. These studies also demonstrated that the effects of electrolytes on bacterial attachment also depended on the bacterial species investigated with different bacterial species reacting to electrolytes differently. This was also observed with the results obtained in these studies.

The lack of cations in a growth medium have been shown to slow down the growth of bacteria and make the cell wall more fragile (Fontana, 1979). These electrolytes could affect bacterial physiology, Na^{+} and Mg^{++} are both involved in specific physiological activities. Changes in bacterial physiological activities can lead to changes in the bacterial cell surface characteristics, which could influence bacterial attachment (Chapter 4). If electrolytes become involved with electrostatic interactions with molecules on the bacterial surface, this could affect the short range interactions between the bacterium and the surface (Chapter 1). This would have an effect on the permanent attachment of the bacterium to the surface.

Evidence is also available to suggest electrolytes can effect the stereochemistry of the bacterial surface. The changes in the attachment of a Streptococcus species, due to the presence of electrolytes, were thought to be due to changes in the polymer coat of the bacterium or due to changes in the charge density of the polymer coat, due to the electrolytes (Rutter, 1978). Studies involving Pseudomonas demonstrated that an increase in cation concentration caused a progressive decrease

in microbial attachment. This was not due to changes in the 1/K but due to the cations interacting with the bacterium's acidic polysaccharide and causing a decrease in bacterial attachment (Fletcher, 1979). Fletcher (1980) also demonstrated a Pseudomonas's adhesive polysaccharide interacting with Al^{3+} to reduce bacterial attachment. The effects of electrolytes on bacterial polymers could influence bacterial attachment as polmers can be important in the attachment of bacteria to surfaces (Chapter 1).

The importance of 1/K on the attachment of bacteria is complex and will be influenced by factors such as the electrolyte and bacterial species present. Chapter 1 discussed the importance of long range and short range forces on the reversible and irreversible attachment of bacteria to surfaces. 1/K is a long range force which could be important in increasing the potential of bacteria attaching irreversibly by increasing the number of bacteria close to the surface. The number of bacteria in close proximity to the surface can be influenced by many factors already discussed (Chapters 3 and 4). Therefore, short range interactions could be the forces of most importance to irreversible attachment e.g. ionic and hydrophobic (Chapter 1), and the importance of other forces will depend on the bacterium which is attaching and the environmental and nutritional conditions present during attachment.

CHAPTER SIX

ATTACHMENT OF A BACTERIUM WHEN IN A MIXED SUSPENSION WITH ANOTHER BACTERIUM.

6.1 AIMS

To study the attachment of the bacteria used in Chapters 4 and 5 when in mixed suspensions with one other bacterium. The attachment of these bacteria were investigated under the same nutritional and environmental parameters used in Chapters 4 and 5.

6.2 INTRODUCTION

As attachment abilities of bacteria have been shown to vary with the growth conditions (Chapters 4 and 5), if two or more bacteria are present in the attachment medium, changing the growth conditions during attachment could alter the relative levels of bacterial species attaching to the solid surface. This could result in the bacterial composition of a biofilm changing if the growth conditions present during attachment were altered.

Little if any research has taken place on the effect of growth conditions on the attachment of bacteria in mixed suspensions. In these studies attachment experiments were performed under the different growth conditions used in Chapters 4 and 5 to examine if the bacteria in mixed suspensions attached in similar proportions as bacteria attached in pure culture. The influence of the growth conditions on the resultant attached bacteria were also investigated to see if changing growth conditions can influence which bacterium in mixed suspensions became dominant on a surface.

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6.3 MATERIALS AND METHODS

6.3.1 Bacteria and Inoculation

The stock cultures of Aeromonas, Chromobacterium, the coryneform and Staphylococcus prepared in Sections 4.3.1 and 5.3.1 were used in these experiments. Batch cultures were inoculated with 1ml of a stock culture and incubated as in Section 3.3.1. 10ml volumes of these stock cultures were used to inoculate the continuous culture apparatus and these continuous culture systems were run as in Section 4.3.1.

6.3.2 The Effect of Carbon Source, Carbon Concentration and Carbon-to-Nitrogen Ratio on the Attachment of Bacteria in Mixed Suspensions.

Aeromonas, Chromobacterium, the coryneform and Staphylococcus were grown in batch culture and continuous culture in the different media outlined in Section 4.3.2. In the case of the bacteria grown in continuous culture a bacterial sample was first removed from the continuous culture apparatus and placed in a sterile 100ml flask.

The number of bacteria present in these 100ml flasks was estimated in Section 3.3.3, hence, approximately equal numbers of the different bacteria could be aseptically mixed together in a sterile 100ml flask. The bacteria present in these mixed suspensions were still in their original growth medium. The total volume of medium in the flask, once the mixed suspension was prepared, was 100ml. The mixed suspensions were placed in a shaking incubator for 10 mins to allow the bacteria to recover from the mixing process. After 10 mins attachment experiments were performed with these mixed bacterial suspensions, as in Section 4.3.3.

6.3.3 Effect of Temperature on the Attachment of Bacterial in Mixed Suspensions

The effect of temperature on the attachment of bacteria in mixed suspensions was investigated by first growing three samples of each bacterium Aeromonas. Chromobacterium. the coryneform and Staphylococcus in media (4), (5) and (6) as in Section 4.3.4. Mixed suspensions of the required bacteria were then prepared. These suspensions were placed in a shaking incubator at the attachment temperatures of 15°C, 25°C or 37°C for 10 mins to allow bacteria to recover from the mixing process. After 10 mins attachment experiments were performed with these mixed bacterial suspensions as in Section 4.3.3.

6.3.4 pH and Attachment

Aeromonas. the coryneform and Staphylococcus were grown in batch culture in Medium (6) at 15°C until late stationary phase as in Section 4.3.2. The bacterial cells were harvested by centrifugation at 11,000 av.g for 15 mins and washed twice with 0.01M Hepes buffer (pH 7.4), as in Section 5.3.2. Mixed suspensions of the bacteria were prepared as the bacteria were resuspended in the mixed buffer system (Section 5.3.2).

Once the bacterial cells had been resuspended in mixed suspensions, they were incubated in a shaking incubator at 15°C for 10 mins to, stabilise them to their new conditions. Glass surfaces were aseptically added to the flasks and attachment experiments were then performed as in Section 4.3.3.

6.3.5 Effect of Electrolyte Type and Concentration on the Attachment of Bacteria in Mixed Suspensions

Aeromonas. the coryneform and Staphylococcus were grown in batch culture in Medium (6) and harvested and washed as in Section 5.3.2. The

bacteria were then resuspended in a series of electrolyte solutions as described in Section 5.3.3 to give the mixed bacterial suspensions required. The resuspended mixed bacterial suspensions were incubated in a shaking incubator at 15°C to stabilise them to the new conditions.

Glass surfaces were aseptically added to the flasks and attachment experiments were performed as in Section 4.3.3. After attachment the surfaces were washed in 0.01M Hepes pH 7.4 instead of phosphate buffer before fixing as this was the main component of the attachment medium.

6.4 RESULTS

Appendix FIGURE 2 (a) and (b) gives examples of the types of biofilms obtained during the attachment experiments in this chapter.

6.4.1 Effect of Growth Conditions on the Attachment of Bacteria in Mixed Suspensions

As with the attachment of pure cultures (FIGURE 4.2 (a)), growth conditions had a profound effect on bacterial attachment when one species was in a mixed suspension with another. As with pure cultures, the bacterial attachment observed depended on the bacterial species investigated e.g. the attachment patterns observed for Aeromonas in mixed suspensions (FIGURE 6.1) were different from those obtained for Chromobacterium in mixed suspensions (FIGURE 6.2).

In general the effects of changing the carbon source on the attachment of a bacterium in a mixed suspension with one other bacterium, was still dependent on the bacterium being investigated. Attachment of Aeromonas in mixed suspension with one other bacterium, was seen to decrease in the mannose and sucrose media, as the carbon concentration increased. This was opposite to the results obtained for Aeromonas attachment in pure culture. In the glucose media however, the

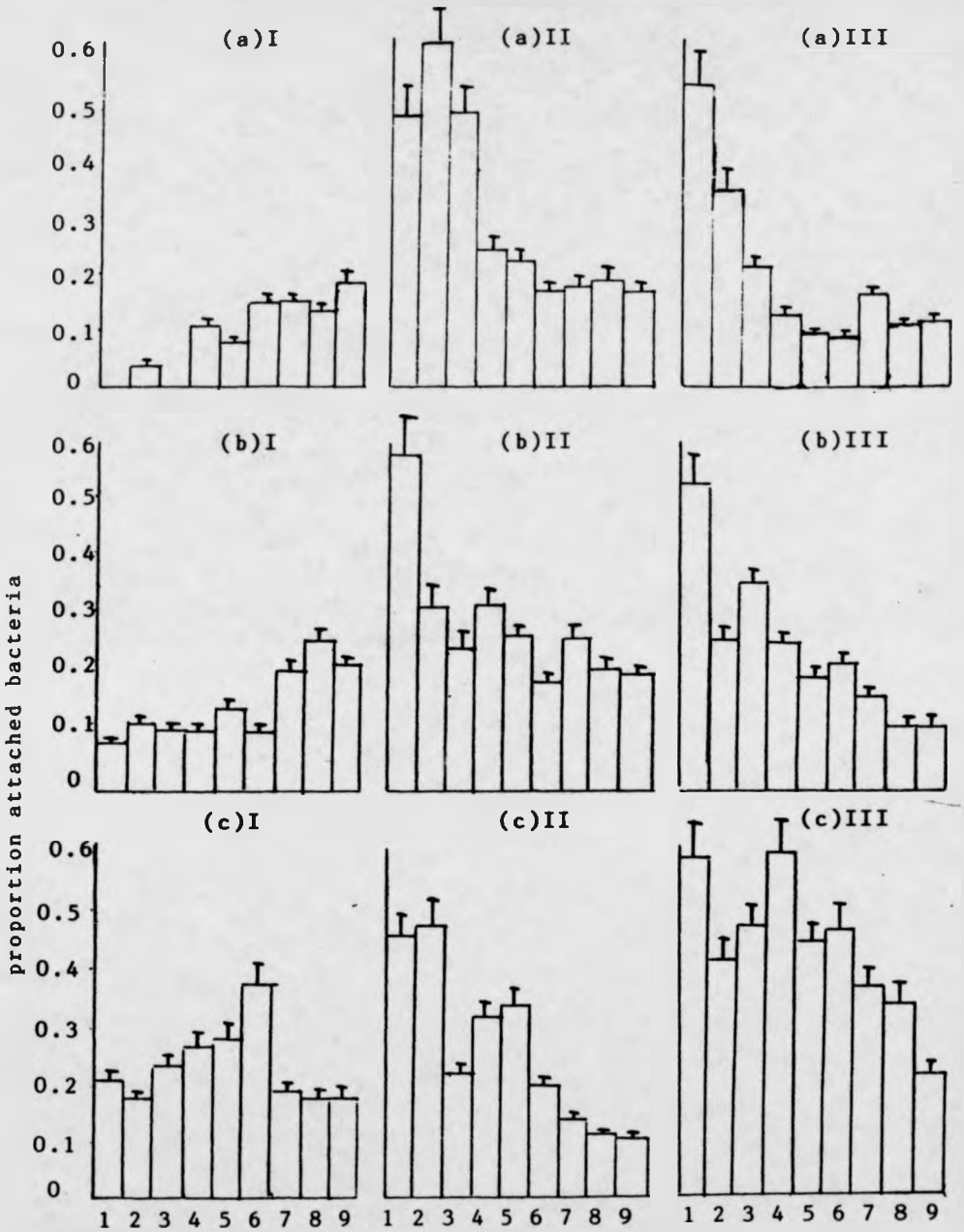


FIGURE 6.1
Attachment of Aeromonas in a mixed suspension with (a) Chromobacterium, (b) the coryneform and (c) Staphylococcus. The carbon sources used in the media were (I) glucose, (II) mannose and (III) sucrose.

N.B.- The media 1-9 are as indicated in the text. The medium listed is the growth medium and the attachment medium.
All cultures were grown in batch culture at 15°C.
The temperature at which attachment was carried out was also 15°C.

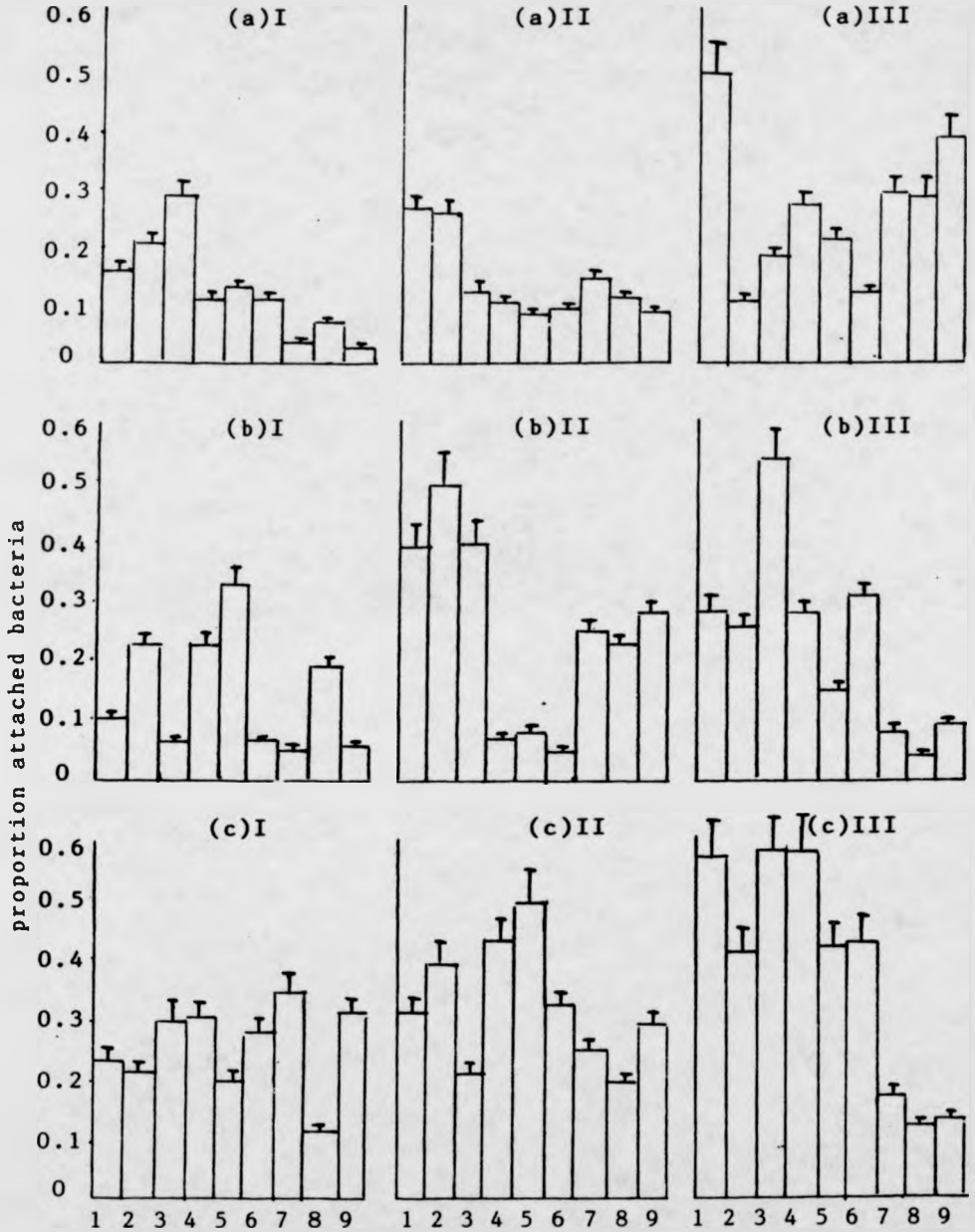


FIGURE 6.2
Attachment of Chromobacterium in a mixed suspension with (a) Aeromonas, (b) the coryneform and (c) Staphylococcus. The carbon sources used in the media were (I) glucose, (II) mannose and (III) sucrose.

N.B.- The media 1-9 are as indicated in the text. The medium listed is the growth medium and the attachment medium. All cultures were grown in batch culture at 15°C. The temperature at which attachment was carried out was also 15°C.

attachment of Aeromonas in mixed suspensions generally increased as the carbon concentration increased. This was similar to the attachment observed with a pure culture. In these experiments, maximum attachment for Aeromonas was observed at carbon concentrations up to 0.02 g carbon l^{-1} compared to 0.2 g carbon l^{-1} with the pure cultures. The effect of carbon-to-nitrogen ratio on the attachment of Aeromonas in mixed suspension varied with the carbon source as for the pure culture results, and with the species present in the mixed suspension e.g. the attachment of Aeromonas in the mannose-limited Medium (1) with the coryneform in the mixed suspension (FIGURE 6.1 (c)II) was greater than the mannose- limited Medium (1) with the Chromobacterium in the mixed suspension.

In general there was no correlation between the attachment of Chromobacterium in mixed suspension with one other bacterium and the carbon concentration (FIGURE 6.2). This was also the case with the pure culture results (FIGURE 4.2 (b)). The attachment of Chromobacterium in mixed suspension was also influenced by the carbon source in the medium. The attachment of Chromobacterium in a mixed suspension with Aeromonas. in glucose medium (FIGURE6.2 (a)I) was different from the attachment of Chromobacterium observed when these species were in a sucrose medium (FIGURE 6.2 (a)III). Changes in the attachment of Chromobacterium were also observed due to the different species in the mixed suspension with Chromobacterium.

As with the attachment of the coryneform in pure culture (FIGURE 4.2 (c)), the attachment of the coryneform in mixed suspension was greater at lower carbon concentrations. These results again were dependent on the carbon source in the media and to a greater extent the bacterium in

the mixed suspension with the coryneform. When Staphylococcus was in a mixed suspension with the coryneform (FIGURE 6.3 (c)), the results were similar to those obtained with the attachment of the pure cultures. However, the attachment observed when Aeromonas or Chromobacterium was in a mixed suspension with the coryneform was greatly decreased if indeed attachment occurred (FIGURE 6.3 (a) and (b)).

The attachment of Staphylococcus in a mixed suspension with Aeromonas or Chromobacterium is hard to assess as little if any attachment of Staphylococcus was observed in the different media used (FIGURE 6.4 (a) and (b)). When Staphylococcus was in a mixed suspension with the coryneform, attachment was observed. The results however, were again dependent on the carbon source in the medium. In the glucose medium (FIGURE 6.4 (c)1) attachment was observed in all of the Media (1) to (9). In the sucrose media however, Staphylococcus attachment was only observed in Media 7 to 9 (FIGURE 6.4 (c)III).

6.4.2 Attachment of Bacteria Grown in Continuous Culture in Mixed Suspensions

As with attachment of pure cultures, changes in the attachment of mixed suspensions of bacteria were observed with continuous culture-grown cells compared to batch culture-grown cells e.g. the attachment of Aeromonas in mixed suspensions when the bacteria were grown in batch culture at 15°C (FIGURE 6.5) was different when the bacteria were grown in continuous culture at 15°C (FIGURE 6.9). The results obtained however, were still dependent on the carbon source as well as the other species present in the mixed suspension.

In general the attachment of Aeromonas in a mixed suspension with Chromobacterium (FIGURE 6.9 (a)) was similar to the attachment observed

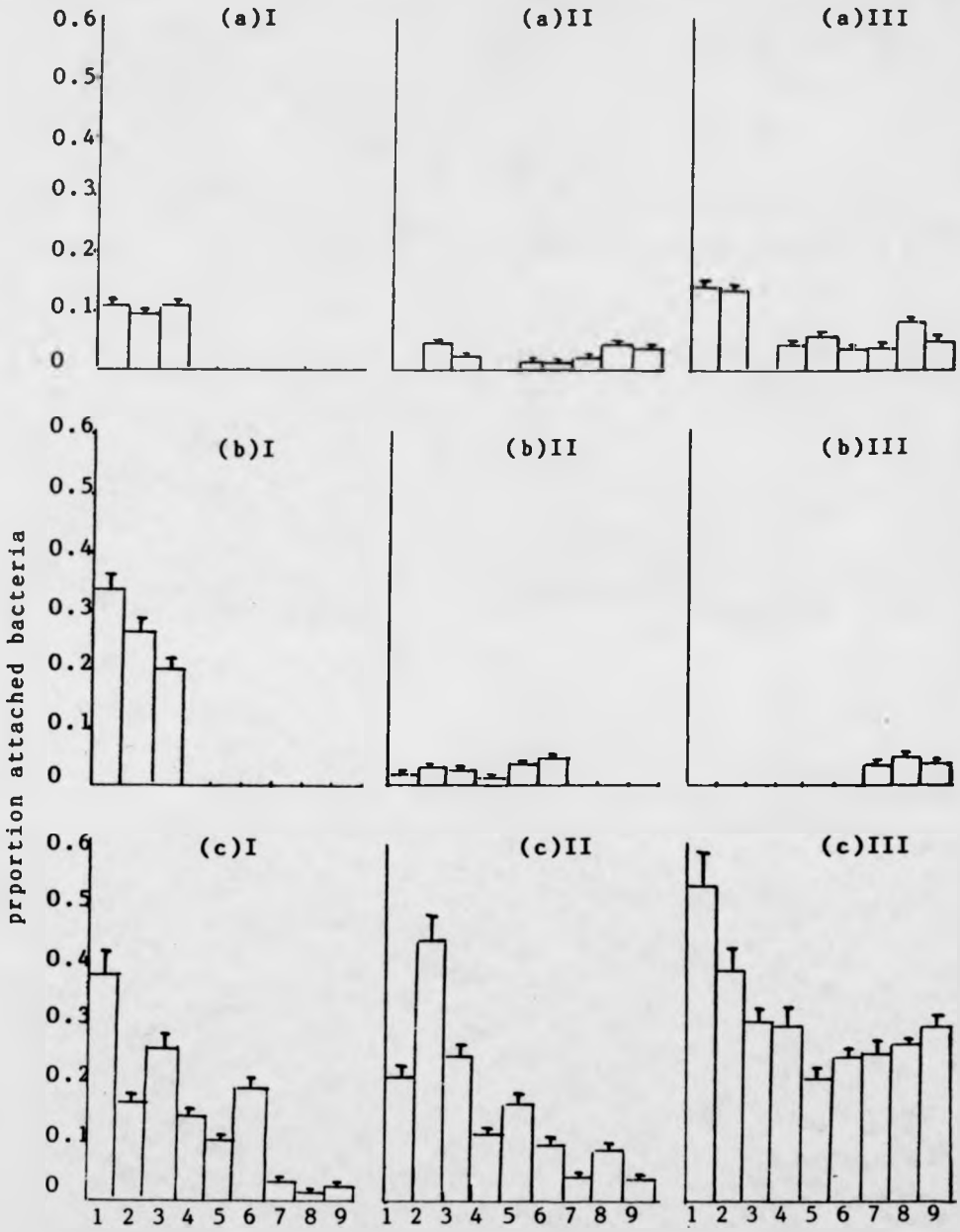


FIGURE 6.3
Attachment of the coryneform in a mixed suspension with (a) Aeromonas, (b) Chromobacterium and (c) Staphylococcus. The carbon sources used in the media were (I) glucose, (II) mannose and (III) sucrose.

N.B.- The media 1-9 are as indicated in the text. The medium listed is the growth medium and the attachment medium.
All cultures were grown in batch culture at 15°C.
The temperature at which attachment was carried out was also 15°C.

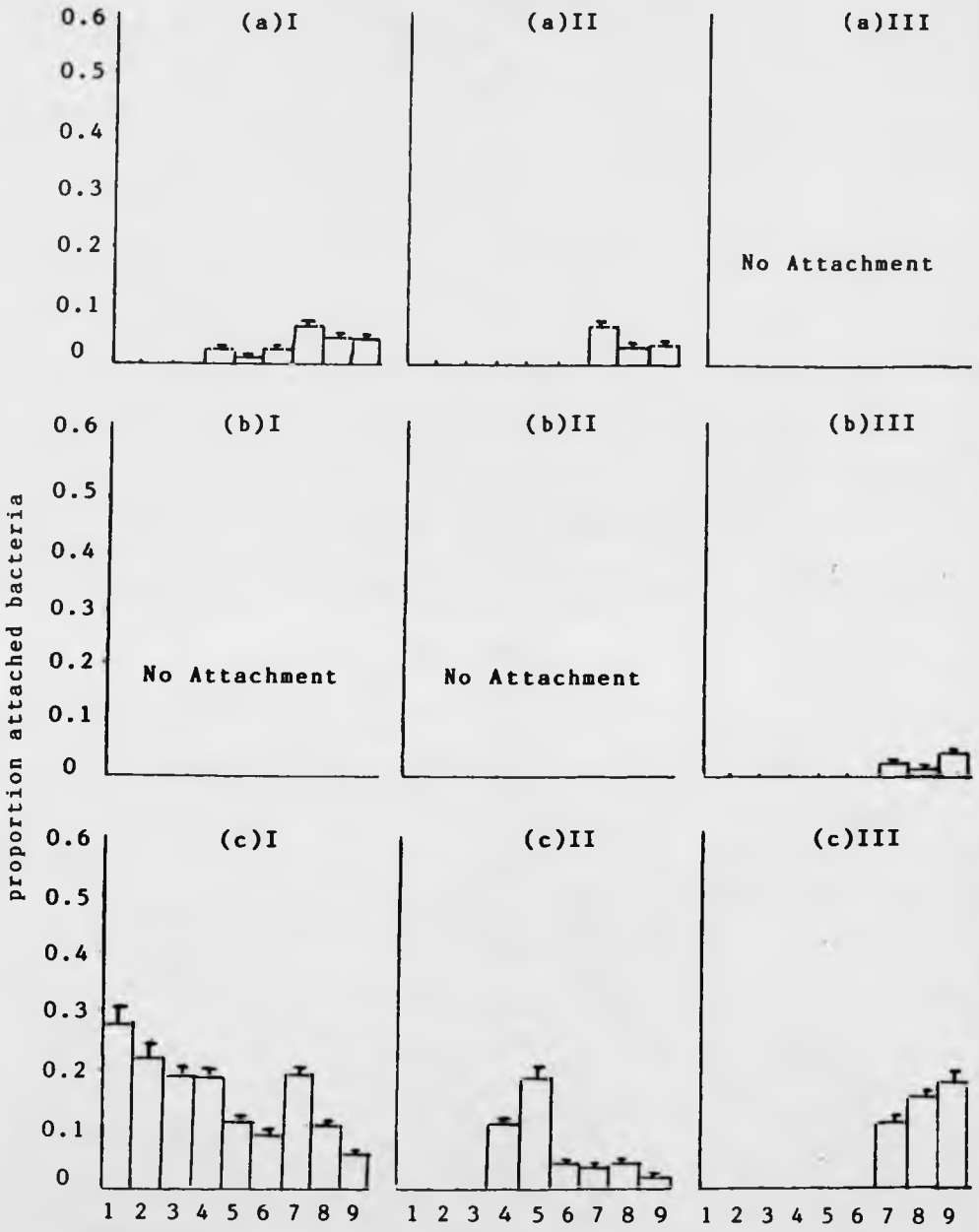


FIGURE 6.4
Attachment of Staphylococcus in a mixed suspension with (a) Aeromonas, (b) Chromobacterium and (c) the coryneform. The carbon sources used in the media were (I) glucose, (II) mannose and (III) sucrose.

N.B.- The media 1-9 are as indicated in the text. The medium listed is the growth medium and the attachment medium.
All cultures were grown in batch culture at 15°C.
The temperature at which attachment was carried out was also 15°C.

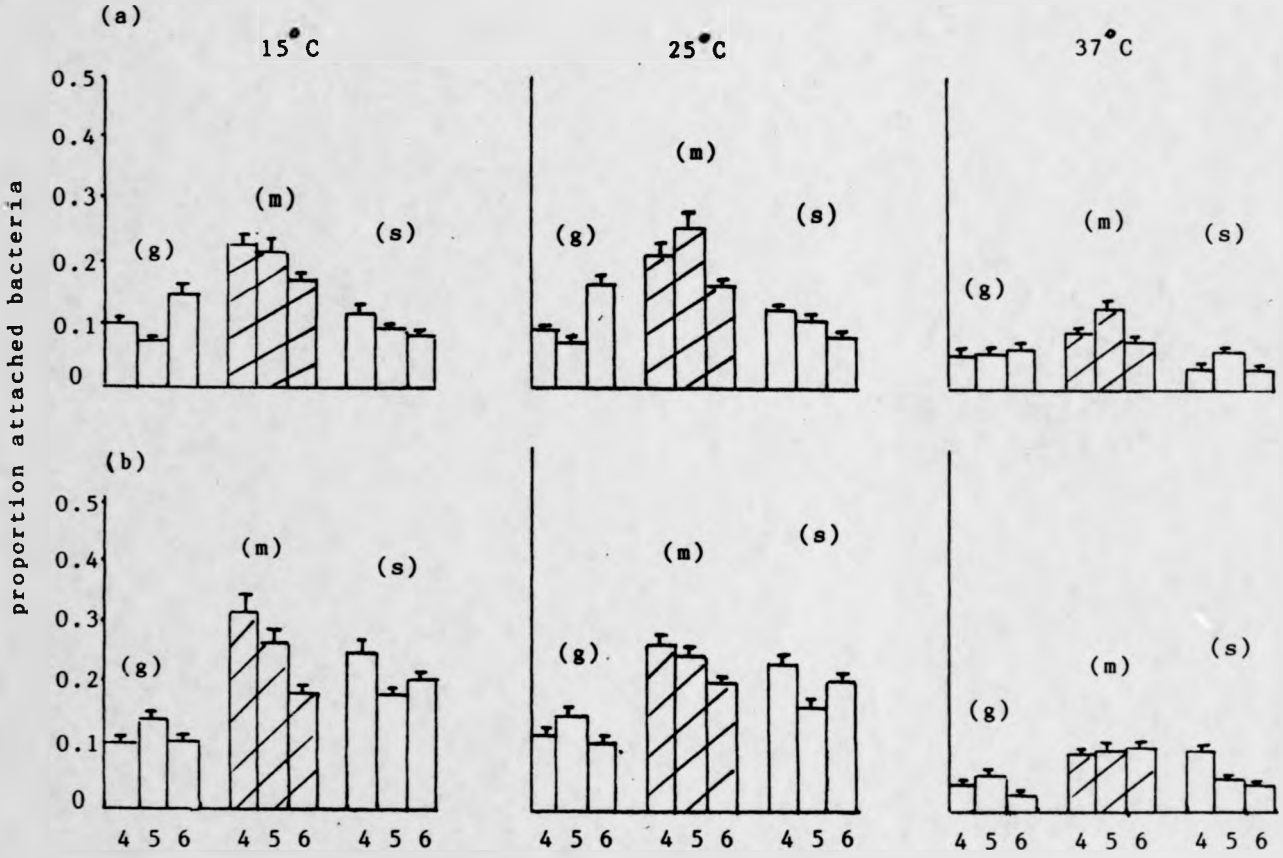


FIGURE 6.5
The attachment of Aeromonas in a mixed suspension with (a) Chromobacterium, (b) the coryneform and (c) Staphylococcus. The carbon sources used in the media were (g) glucose, (m) mannose and (s) sucrose.

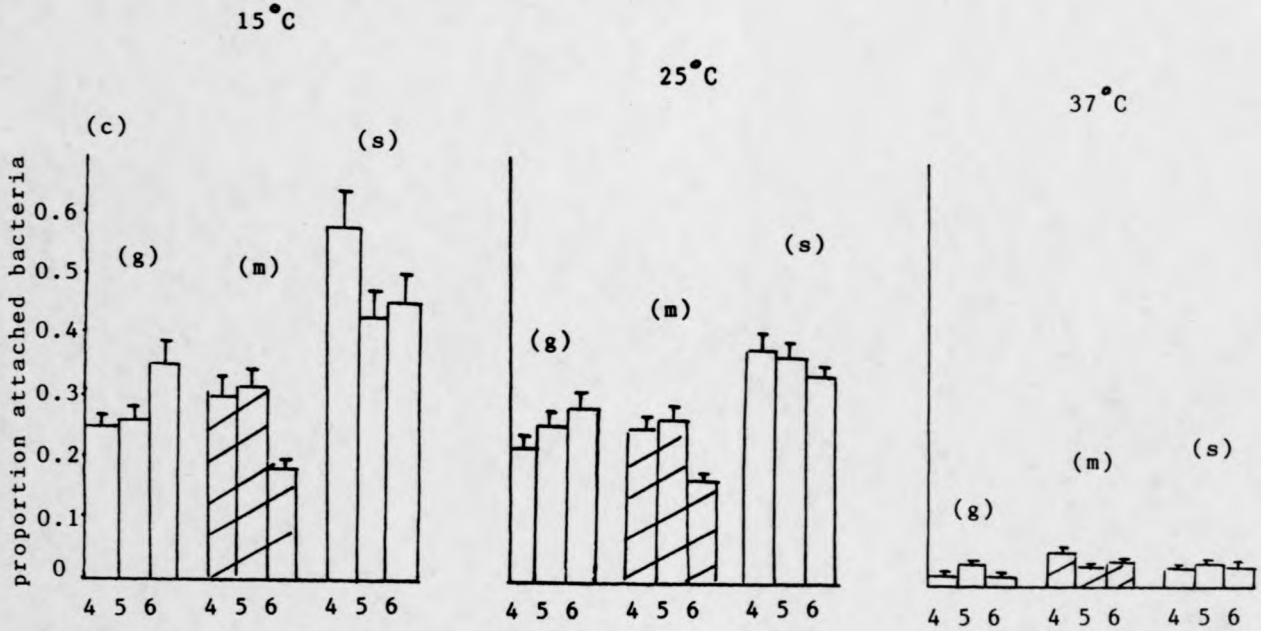


FIGURE 6.5 (continued)

N.B.- The media 4-6 are as indicated in the text. The medium listed is the growth medium and the attachment medium. All culture were grown in batch culture at 15°C. After mixed suspensions were prepared the attachments were performed at the temperatures indicated.

for Aeromonas in pure culture (FIGURE 4.3 (a). When the coryneform was present in the mixed suspension with Aeromonas the attachment of Aeromonas increased compared to the pure culture results (FIGURE 6.9 (b)). Staphylococcus in mixed suspension with Aeromonas again produced similar results to those obtained with the pure culture of Aeromonas.

When Chromobacterium was in a mixed suspension with Aeromonas, the coryneform or Staphylococcus, the attachment (FIGURE 6.10) was always similar to that obtained for the attachment of pure cultures of Chromobacterium (FIGURE 4.3 (b)). Differences in Chromobacterium attachment in mixed suspensions were still apparent, however, compared to pure culture attachment when the carbon-to-nitrogen ratios in the media were investigated.

The coryneform when in mixed suspensions attached to a lesser extent (FIGURE 6.11) compared to the pure culture attachment results (FIGURE 4.3 (c)). When Aeromonas or Chromobacterium were in the mixed suspension with the coryneform (FIGURE 6.11 (a) and (b)) the coryneform did not attach in any of the media. When Staphylococcus was in a mixed suspension with Aeromonas or Chromobacterium (FIGURE 6.12 (a) and (b)) the attachment was always lower compared to the attachment of a pure culture of Staphylococcus (FIGURE 4.3 (d)). When the coryneform was in a mixed suspension with Staphylococcus (FIGURE 6.12 (c)), the attachment of Staphylococcus was greater in the glucose media compared to the attachment of the pure culture in glucose media. In the mannose and sucrose media, the attachment of the Staphylococcus was slightly less than the pure culture attachment.

In summary the results obtained in Sections 6.4.1 and 6.4.2 demonstrate that the growth conditions have a profound effect on the

attachment of bacteria when in a mixed suspension. The attachment results obtained for a bacterium, attached under different growth conditions in pure culture, were often different from the results obtained when the species was attached in a mixed suspension. These results suggested the bacteria present in a mixed suspension could influence the attachment of each other in many instances.

6.4.3 Effect of Temperature on the Attachment of Bacteria in Mixed Suspensions

Aeromonas in a mixed suspension (FIGURE 6.5) attached differently compared to the attachment of a pure culture of Aeromonas (FIGURE 4.4 (a)) at different temperatures. As seen with the attachment of pure cultures of Aeromonas there was little change in the attachment of mixed suspensions of Aeromonas when the attachment temperature was increased from 15°C to 25°C but, a significant decrease in attachment was seen when the temperature was increased to 37°C. This was opposite to the results obtained with the attachment of pure cultures of Aeromonas where an increase was observed when the temperature was increased from 25°C to 37°C. These results were observed whether the Aeromonas was in a mixed suspension with Chromobacterium. the coryneform or Staphylococcus. In general the numbers of Aeromonas attaching was nearly always lower with the mixed suspensions than the attachment observed with the pure cultures.

The results obtained for the effects of temperature on the attachment of Chromobacterium in mixed suspension with Aeromonas. the coryneform and Staphylococcus are shown in FIGURE 6.6. There was again little change in Chromobacterium attachment in mixed suspension with either of the other species when the attachment temperature was

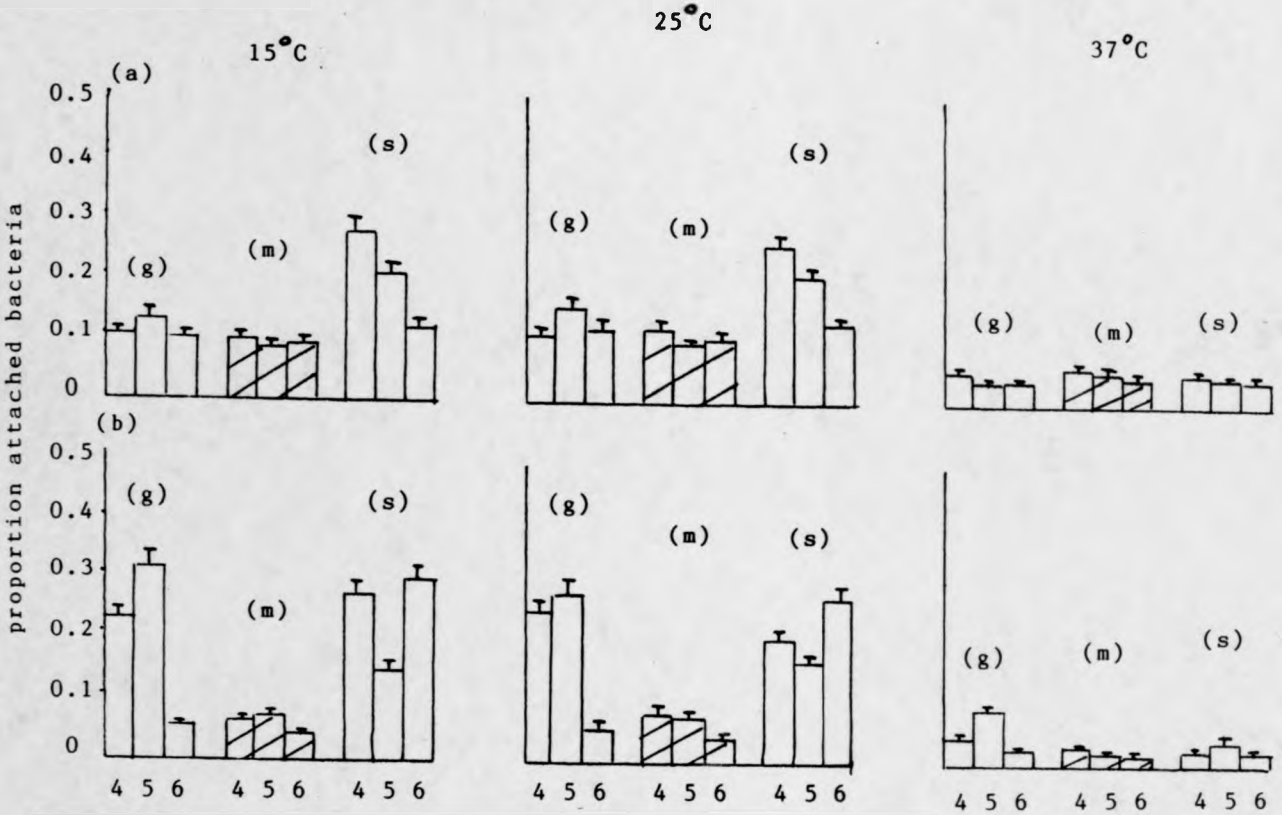


FIGURE 6.6
The attachment of Chromobacterium in a mixed suspension with (a) Aeromonas, (b) the coryneform and (c) Staphylococcus. The carbon sources used in the media were (g) glucose, (m) mannose and (s) sucrose.

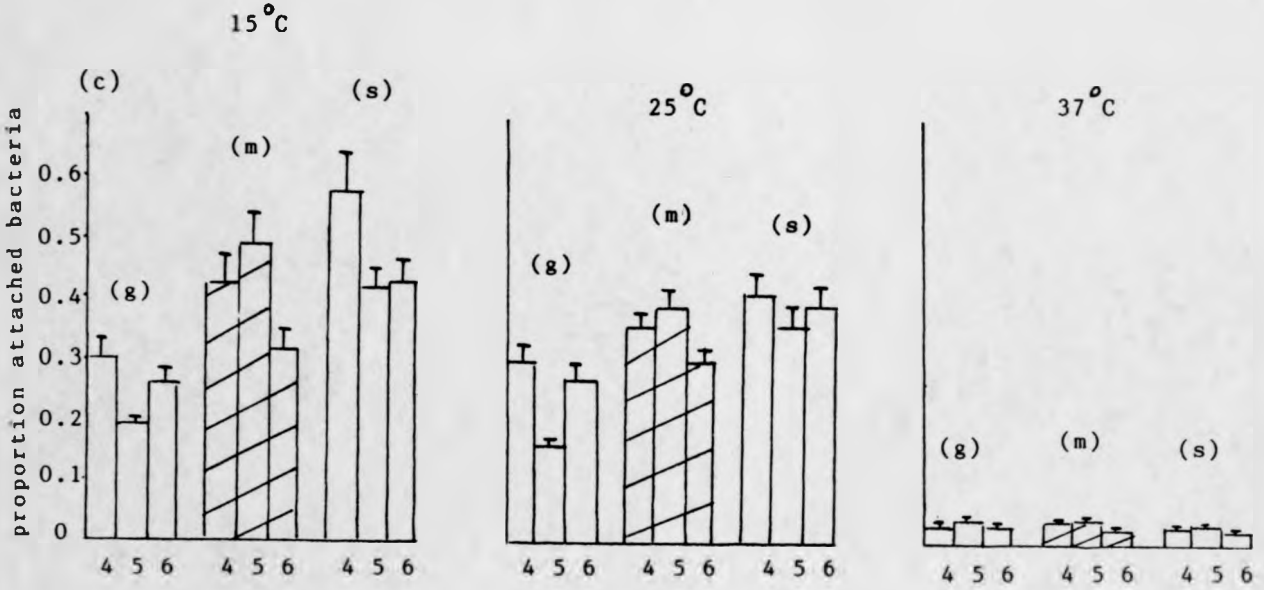


FIGURE 6.6 (continued)

N.B.- The media 4-6 are as indicated in the text, The medium listed is the growth medium and the attachment medium. All cultures were grown in batch culture at 15°C. After mixed suspensions were prepared the attachments were performed at the temperatures indicated.

increased from 15°C to 25°C. These results were again similar to those obtained for the attachment of pure cultures of Chromobacterium (FIGURE 4.4 (b)). Once again when the temperature was increased from 25°C to 37°C the attachment of Chromobacterium in mixed suspensions decreased significantly (P>0.05). This again was the opposite to the results obtained for the attachment of pure cultures of Chromobacterium (FIGURE 4.4 (b)). As was seen with the Aeromonas, the numbers of Chromobacterium attaching in the mixed suspension experiments, was usually lower than in the attachment of pure cultures of Chromobacterium.

The coryneform in mixed suspension with Aeromonas or Chromobacterium (FIGURE 6.7 (a) and (b)) demonstrated little if any attachment at 15°C or 25°C. This was not the case with pure cultures of the coryneform (FIGURE 4.4 (c)) where the coryneform attached at all temperatures. The attachment of the coryneform in mixed suspensions with Aeromonas and Chromobacterium significantly (P>0.05) increased when the temperature was increased from 25°C to 37°C. When the coryneform was in mixed suspension with Staphylococcus, the attachment at the different temperatures was similar to that obtained for Aeromonas and Chromobacterium in mixed suspensions. In this case, the attachment of the coryneform in mixed suspension with Staphylococcus did not change between 15°C and 25°C. When the temperature was increased to 37°C, the attachmemt of the coryneform decreased significantly (P>0.05) from the results obtained at 15°C and 25°C. These results were again different from those obtained for the attachment of pure cultures of the coryneform. The coryneform numbers attaching in mixed suspension with Staphylococcus, were again less than those which attached when the coryneform was in pure culture.

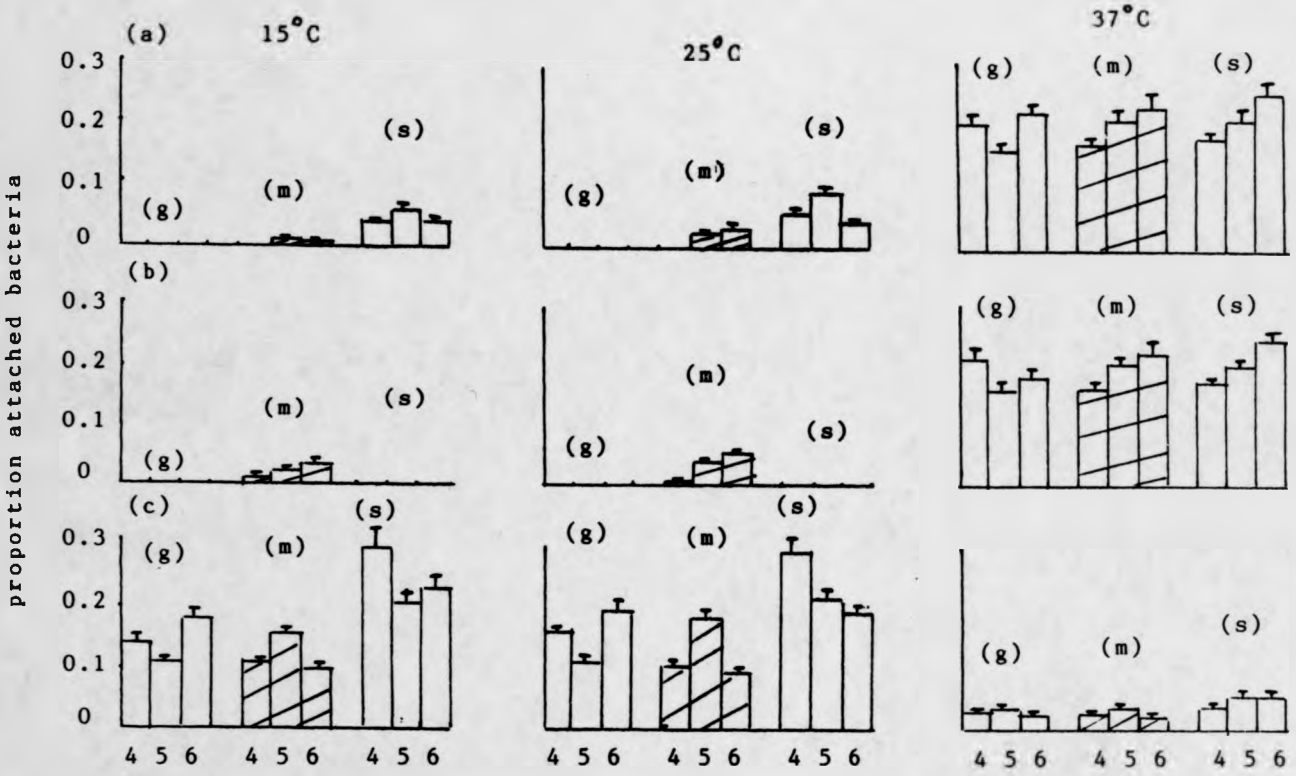


FIGURE 6.7
The attachment of the coryneform in a mixed suspension with (a) Aeromonas, (b) Chromobacterium and (c) Staphylococcus. The carbon sources used in the media were (g) glucose, (m) mannose and (s) sucrose.

N.B.- The media 4-6 are as indicated in the text. The medium listed is the growth medium and the attachment medium. All cultures were grown in batch culture at 15°C. After mixed suspensions were prepared the attachments were performed at the temperatures indicated.

Staphylococcus in mixed suspension with Aeromonas or Chromobacterium also demonstrated little if any attachment at 15°C or 25°C. When the temperature was increased to 37°C the attachment of Staphylococcus was again seen to increase (FIGURE 6.8 (a) and (b)). These results are the opposite of those obtained for attachment of pure cultures of Staphylococcus at different temperatures. In pure culture, the attachment of Staphylococcus decreased when the temperature was increased to 37°C. In suspension with the coryneform, variations in attachment were observed, compared to the other mixed suspension results. In this case, no attachment of Staphylococcus was obtained at 25°C or 37°C. Attachment of Staphylococcus, however, did occur at 15°C. In general these results were not comparable with those obtained for the attachment of Staphylococcus in pure cultures.

The results obtained for the effects of temperature on the attachment of bacteria in mixed suspensions, when the bacterial cells were grown in continuous culture, were similar to those obtained when the bacteria were grown in batch culture. In the case of Aeromonas in a mixed suspension with Chromobacterium. the coryneform and Staphylococcus (FIGURE 6.9) bacterial attachment decreased as the temperature increased from 15°C to 25°C, and from 25°C to 37°C. Similar results were also obtained when Chromobacterium was in mixed suspension with Aeromonas. the coryneform and Staphylococcus (FIGURE 6.10). In these experiments, a decrease in Chromobacterium attachment was mostly apparent when the attachment temperature increased from 25°C to 37°C.

After the coryneform was grown in continuous culture and attached in mixed suspensions with Aeromonas and Chromobacterium. no attachment of the coryneform was observed at any of the attachment temperatures

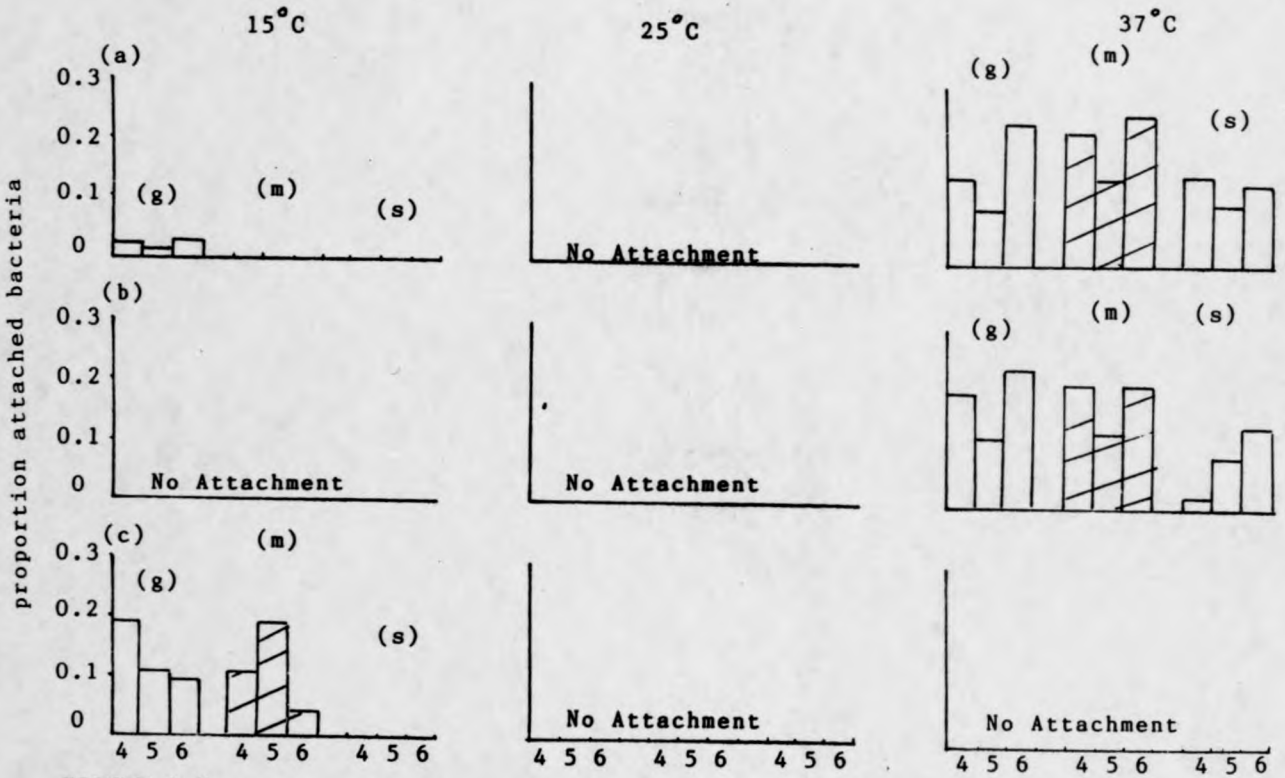


FIGURE 6.8
The attachment of Staphylococcus in a mixed suspension with (a) Aeromonas, (b) Chromobacterium and (c) the coryneform. The carbon sources used in the media were (g) glucose, (m) mannose and (s) sucrose.

N.B. - The media 4-6 are as indicated in the text. The medium listed is the growth medium and the attachment medium. All cultures were grown in batch culture at 15°C. After mixed suspensions were prepared the attachments were performed at the temperatures indicated.

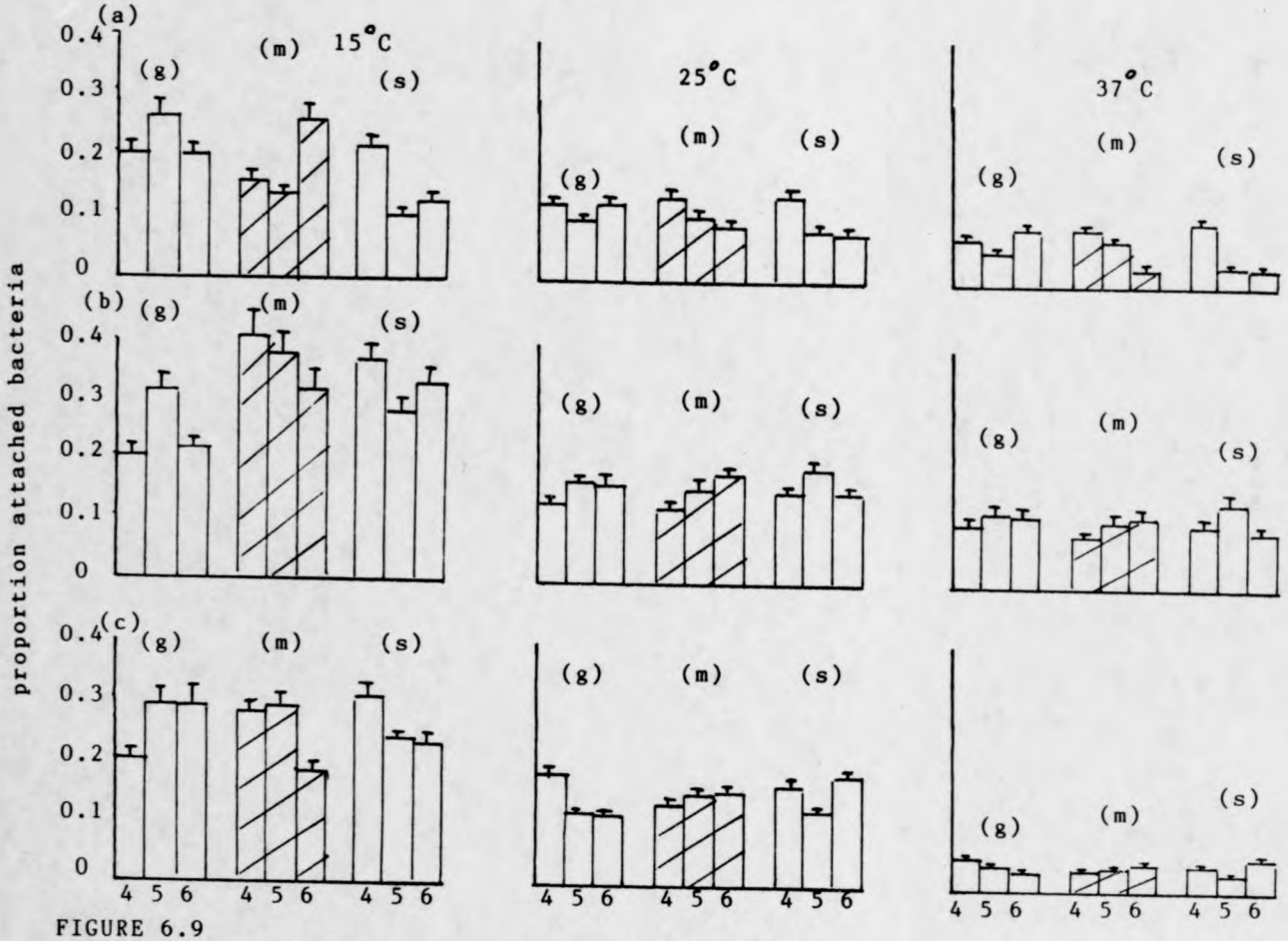


FIGURE 6.9
Attachment of Aeromonas in a mixed suspension with (a) Chromobacterium, (b) the coryneform and (c) Staphylococcus. The carbon sources used in the media were (g) glucose, (m) mannose and (s) sucrose.

N.B.- The medium listed is the growth medium and the attachment medium. All cultures were grown in continuous culture at 15°C. Attachments were performed at the temperatures indicated.

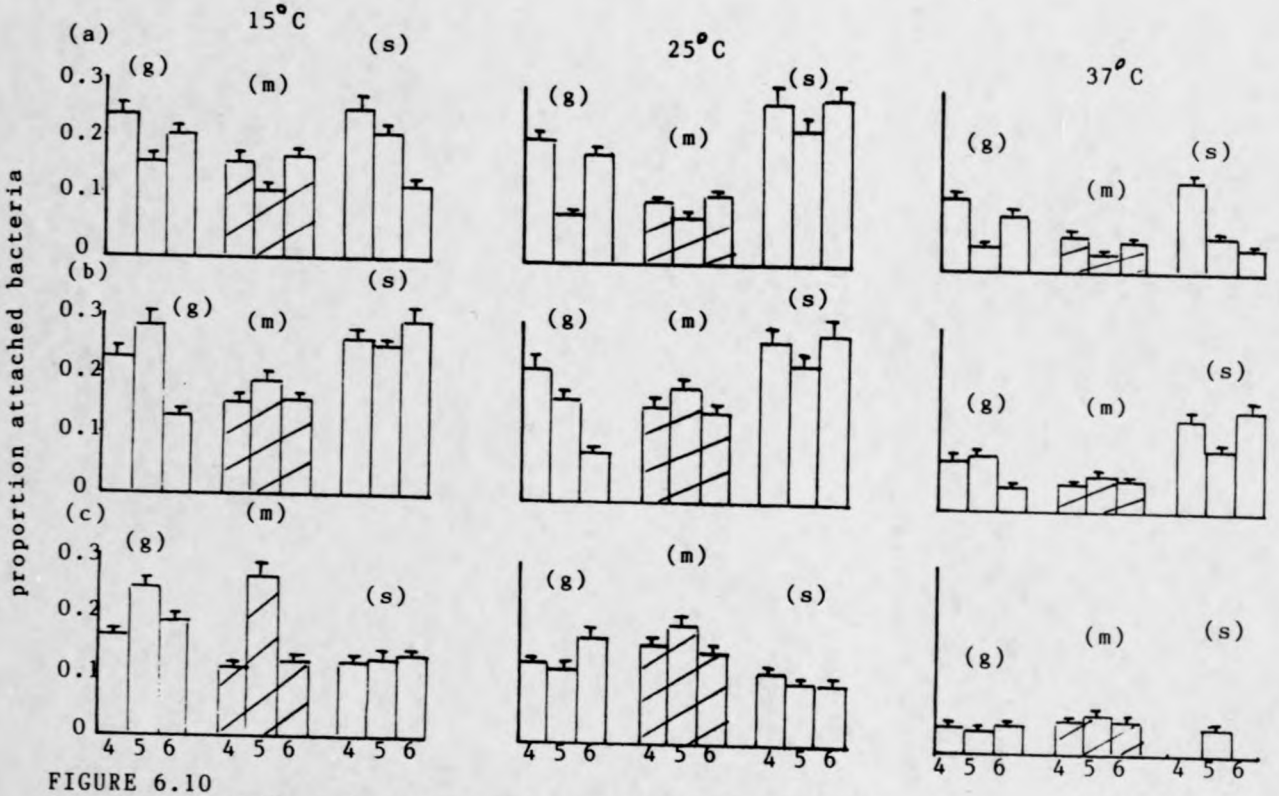


FIGURE 6.10
Attachment of Chromobacterium in a mixed suspension with (a) Aeromonas, (b) the coryneform and (c) Staphylococcus. The carbon sources used in the media were (g) glucose, (m) mannose and (s) sucrose.

N.B- The medium listed is the growth medium and the attachment medium. All cultures were grown in continuous culture at 15°C. Attachments were performed at the temperatures indicated.

(FIGURE 6.11 (a) and (b)). These results were different from those obtained with the batch culture grown cells. When the coryneform was in mixed suspension with Staphylococcus, after both bacteria had been grown in continuous culture, little change in the coryneform attachment was observed when the temperature was increased from 15°C to 25°C (FIGURE 6.11 (c)). As the temperature was increased to 37°C, however, a decrease in the coryneform attachment was observed, similar to the results obtained with cells grown in batch culture.

Staphylococcus, grown in continuous culture and attached in mixed suspensions with Aeromonas, Chromobacterium and the coryneform, attached in all the media tested and at all temperatures (FIGURE 6.12). This was not seen when Staphylococcus was grown in batch culture. Similarities, however, were seen with the cells grown in batch culture when investigating Staphylococcus attachment in mixed suspension with Aeromonas or Chromobacterium. In these cases little change in attachment was observed between 15°C and 25°C. As the temperature increased to 37°C, an increase in Staphylococcus attachment was observed.

In general, temperature could still influence attachment of a mixed suspension of bacteria as was seen with attachment of pure cultures of bacteria. However, as was seen in Sections 6.4.1 and 6.4.2 the results obtained were dependent upon the bacteria being investigated and the other species in the mixed suspension.

6.4.4 The Effect of pH on the Attachment of Bacteria in Mixed Suspensions

When the attachment of Aeromonas, the coryneform and Staphylococcus was studied over a range of pH's it was clear that the results obtained were different from the attachment results for the attachment of pure

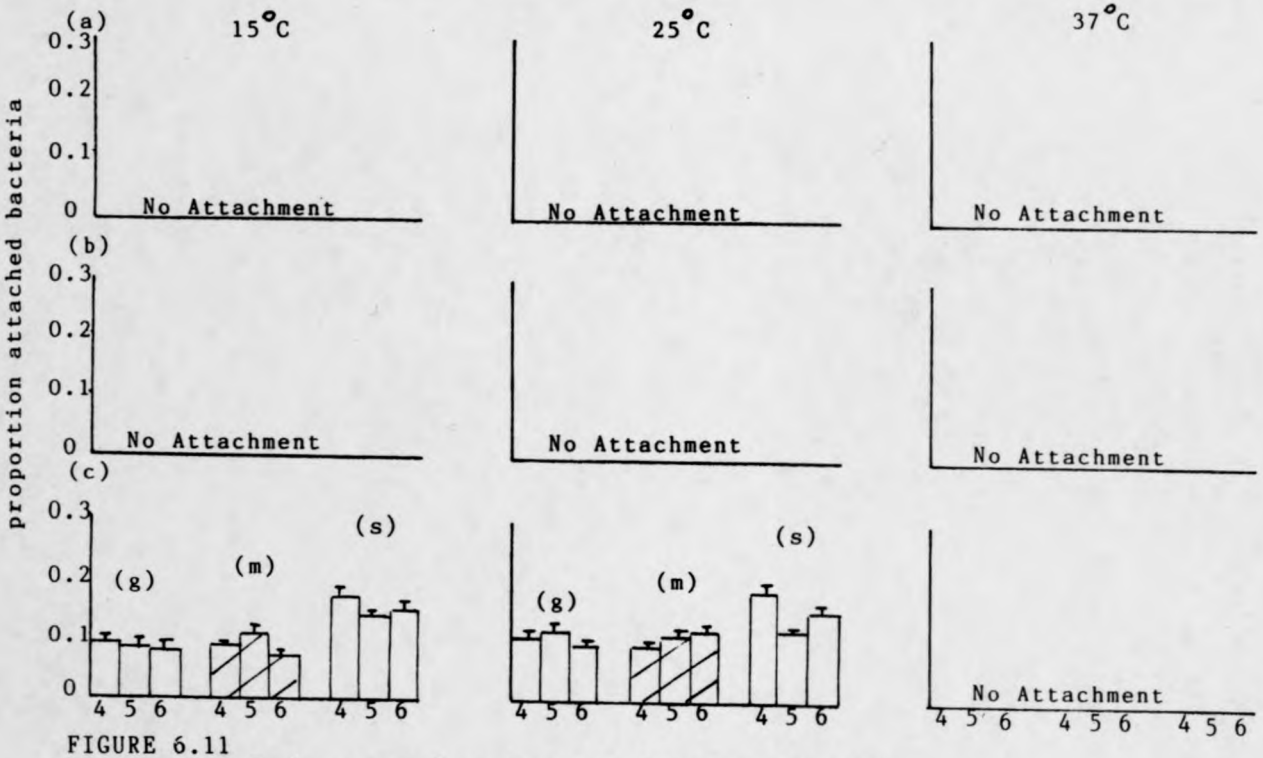


FIGURE 6.11
Attachment of the coryneform in a mixed suspension with (a) Aeromonas, (b) Chromobacterium and (c) Staphylococcus. The carbon sources used in the media were (g) glucose, (m) mannose and (s) sucrose.

N.B.- The medium listed is the growth medium and the attachment medium. All cultures were grown in continuous culture at 15°C. Attachments were performed at the temperatures indicated.

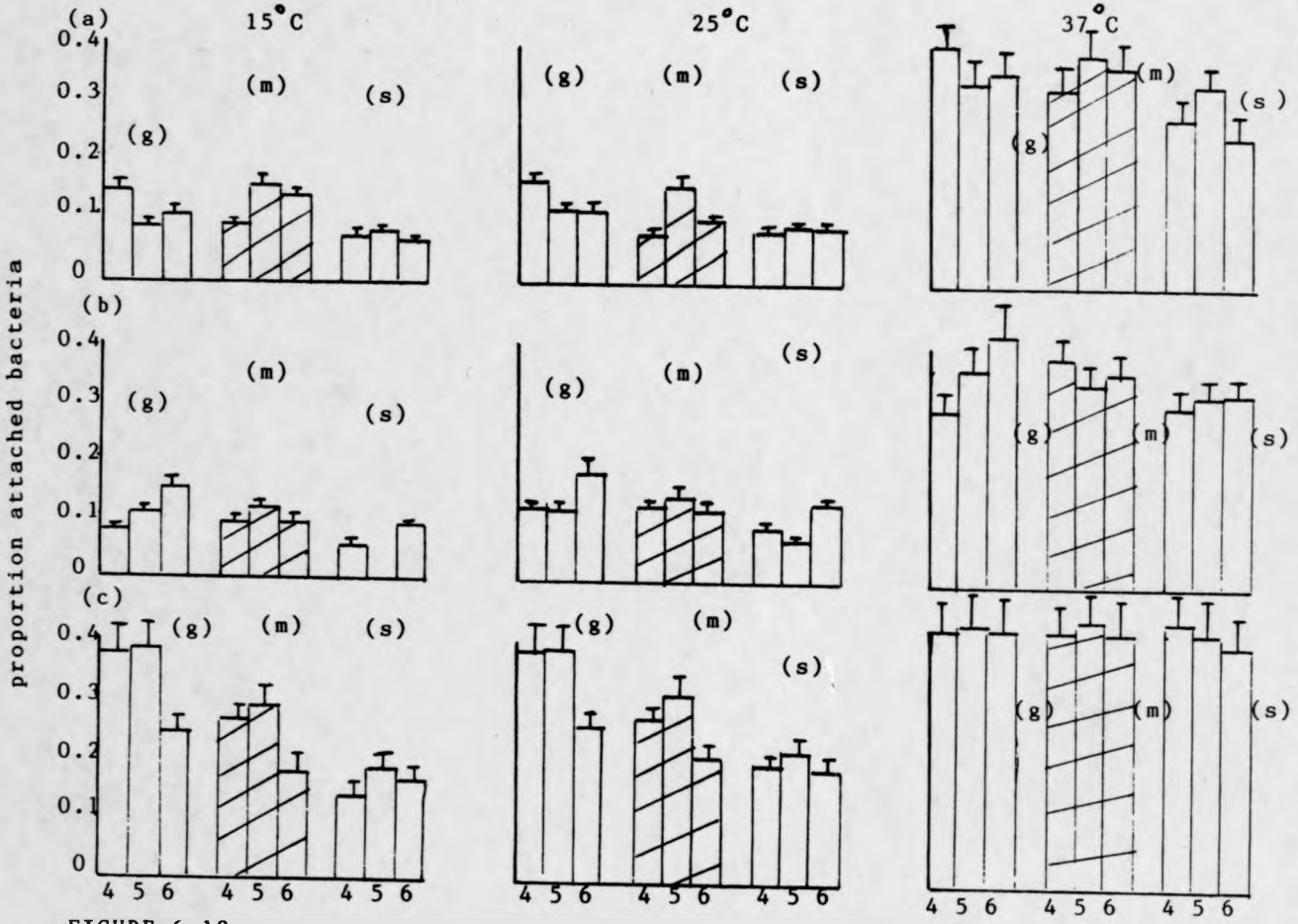


FIGURE 6.12
Attachment of Staphylococcus in a mixed suspension with (a) Aeromonas. (b) Chromobacterium and (c) the coryneform. The carbon sources used in the media were (g) glucose, (m) mannose and (s) sucrose.

cultures of bacteria. The attachment of pure cultures of Aeromonas gave an optimum attachment pH between 7.5 and 8.0 (FIGURE 5.3 (a)). When Aeromonas was in a mixed suspension (FIGURE 6.13), the pH optimum for attachment changed. The pH optimum obtained was dependent on the bacterium in the mixed suspension with Aeromonas. The pH optimum when the coryneform was in the mixed suspension with Aeromonas (FIGURE 6.13 (a)) was between pH 7 and pH 7.5. The overall bacterial attachment with the mixed suspension bacteria was greater at this optimum than the figure obtained for the pure culture bacteria. Aeromonas was also found to attach over the full pH range when the coryneform was present in the mixed suspension. This was not seen with Aeromonas attachment in pure cultures where no attachment occurred at pH 4.0 or pH 4.5.

Aeromonas in a mixed suspension with Staphylococcus (FIGURE 6.13 (b)) attached at a pH optimum different from that of the pure culture, and from that of Aeromonas attachment in mixed suspension with the coryneform. The optimum pH for attachment in this case was pH 6.5. Aeromonas was again seen to attach in greater numbers at this optimum than observed with the pure culture. Aeromonas attached over the full pH range when Staphylococcus was present in the mixed suspension even though Aeromonas did not attach at pH 4 or pH 4.5 in pure culture. These results over the full pH range were similar to those obtained when the coryneform was in the mixed suspension with Aeromonas.

The coryneform in mixed suspension with Aeromonas and Staphylococcus over a pH range gave results which were again different from the attachment of the coryneform in pure culture (FIGURE 5.3 (b)). When Aeromonas was in the mixed suspension with the coryneform, no attachment of the coryneform occurred. Attachment did, however, take place when

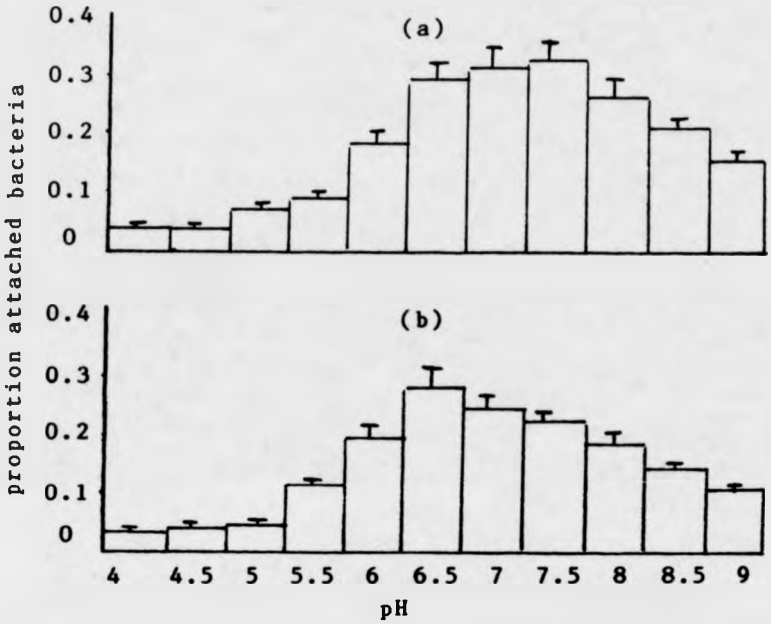


FIGURE 6.13
The attachment of (a) Aeromonas in a mixed suspension with the coryneform and (b) Aeromonas in a mixed suspension with Staphylococcus in a mixed buffer system.

N.B.- The buffer system was adjusted to pH's from 4.0 to 9.0. The attachments were performed in this buffer system in a shaking incubator at 15°C.

The bacteria were first grown in batch culture in Medium 6 at 15°C. The bacteria were harvested and resuspended to produce the mixed suspensions required in the above mixed buffer system.

Staphylococcus was in the mixed suspension with the coryneform (FIGURE 14). The coryneform demonstrated a similar pH optimum for attachment in pure culture (pH 6.5 to pH 7) and in mixed suspension (pH 6.5). The number of bacteria which attached at the pH optimum for the mixed suspension was again greater than the number of bacteria which attached at the pH optimum for the pure culture.

The effect of pH on the attachment of Staphylococcus in mixed suspensions with Aeromonas or the coryneform, was slightly different from the effects observed for the other mixed suspensions. Staphylococcus. in mixed suspension with Aeromonas, again did not attach over the pH range. Staphylococcus however, did attach over the pH range when in a mixed suspension with the coryneform (FIGURE 6.15). The pH optimum for the attachment of Staphylococcus in pure culture was pH 6 (FIGURE 5.3 (c)), and in mixed suspension with the coryneform, the optimum was pH 7.5. In this case, the degree of attachment at the pH optimum of Staphylococcus when in mixed suspension was lower than the attachment observed with the pure culture. Staphylococcus in mixed suspension with the coryneform, attached at pH 5 or above, however, in pure culture Staphylococcus did not attach at pH 8.5 or pH 9.

In general the results show that the bacterial mixed suspensions reacted to pH variations differently to the bacteria in pure culture. When a mixed suspension was used, however, the bacterium present in the mixed suspension influenced the reaction of the other species in the mixed suspension to pH changes. In the case of Aeromonas attachment, attachment was seen over the whole pH range when Aeromonas was in a mixed suspension. Pure cultures of Aeromonas, however, did not attach

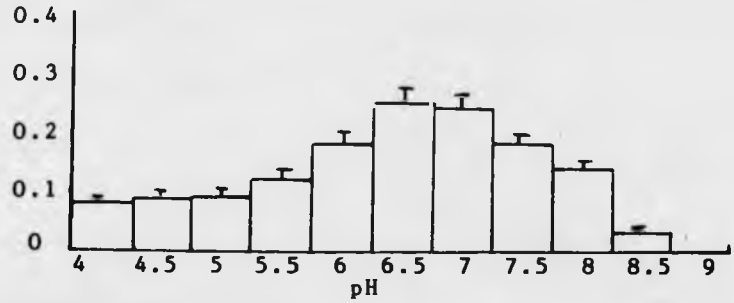


FIGURE 6.14
The attachment of the coryneform in a mixed suspension with Staphylococcus in the mixed buffer system.
The coryneform did not attach in the mixed buffer system when in a mixed suspension with the Aeromonas.

proportion attached bacteria

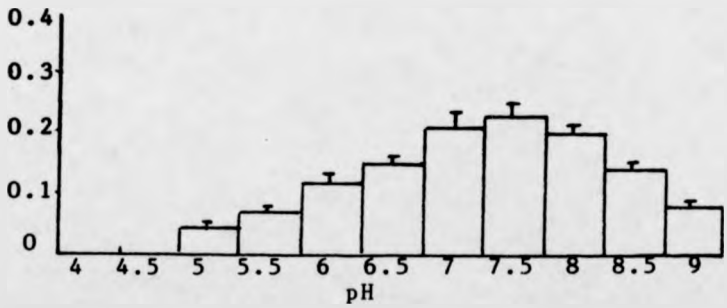


FIGURE 6.15
The attachment of Staphylococcus in a mixed suspension with the coryneform in the mixed buffer system.
The Staphylococcus did not attach in the mixed buffer system when in a mixed suspension with the Aeromonas.

N.B.- The buffer system in FIGURES 6.14 and 6.15 was adjusted to pH's from 4.0 to 9.0. The attachments were performed in this buffer system in a shaking incubator at 15°C.

The bacteria were first grown in batch culture in Medium 6 at 15°C. The bacteria were harvested and resuspended to produce the mixed suspensions required in the above mixed buffer system.

over the full pH range. This was not the case with the coryneform which attached over the full pH range in pure culture and in mixed suspension.

6.4.5 The Effect of Electrolyte Type and Concentration on the Attachment of Bacteria in Mixed Suspension

The effects of 1/K on the attachment of pure cultures of Aeromonas and Aeromonas in a mixed suspension with the coryneform and Staphylococcus are presented in FIGURE 6.16. In all cases, Aeromonas attached in greater numbers in pure culture than in mixed suspension. In the pure culture, (FIGURE 6.16 (a)) Aeromonas attached to a significantly greater extent ($P>0.05$) when the 1/K was 0.3 than in the control. This was also the case when Aeromonas was in a mixed suspension with the coryneform (FIGURE 6.16 (b)). When the 1/K value was 1.5 Aeromonas attached less than the control. This was the case whether Aeromonas was in pure culture or in a mixed suspension with the coryneform. If the 1/K value was 1.0, the results obtained with Aeromonas in pure culture and with Aeromonas in mixed suspension with the coryneform were not comparable as Aeromonas attached differently in each case.

In mixed suspension with Staphylococcus, Aeromonas was again seen to attach differently compared with the pure culture of Aeromonas. This can be seen when the 1/K value is 3.1, in pure culture the attachment of Aeromonas was greater than the control value. When Aeromonas was in a mixed suspension with Staphylococcus the attachment was lower than the control value.

As discussed for the pure culture results, the salt present in the attachment solution could influence the attachment results obtained. This can be seen in pure culture where Aeromonas attachment in the Na

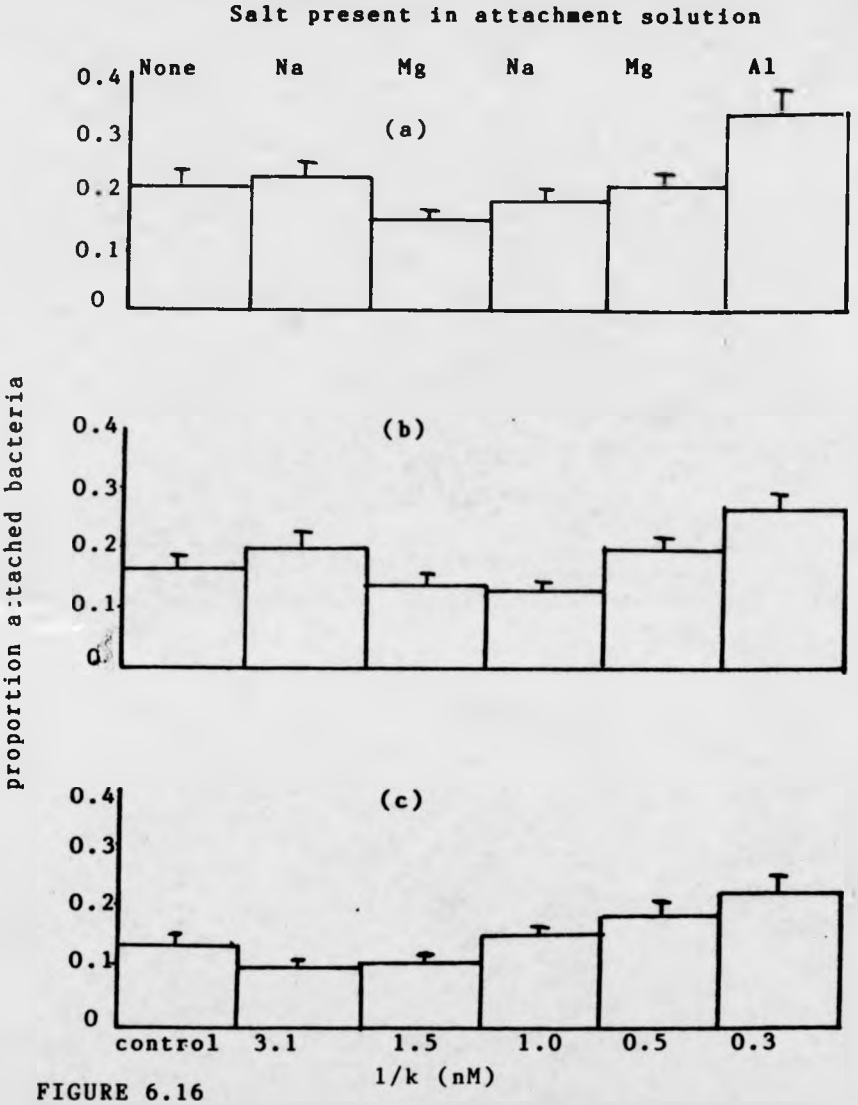


FIGURE 6.16
The effect of the electrical double-layer thickness on the attachment of (a) Aeromonas in pure culture, (b) Aeromonas in a mixed suspension with the coryneform and (c) Aeromonas in a mixed suspension with Staphylococcus.

N.B.- The bacteria were attached in solutions with different electrolyte types and concentrations. These solutions were incubated in a shaking incubator at 15°C.

The bacteria were first grown in batch culture in Medium 6 at 15°C. The bacteria were harvested and resuspended to produce the mixed suspensions required in the electrolyte solutions.

salt media was greater at the lowest 1/K value. This was also the case when Aeromonas was in a mixed suspension with the coryneform, but was not the case when Aeromonas was in a mixed suspension with Staphylococcus. Therefore, the 1/K value or the salt present in the attachment solution could be influencing the attachment of Aeromonas. The results obtained in turn could be influenced by the species present in the mixed suspension with Aeromonas.

The effects of 1/K on the attachment of the coryneform in pure culture and in mixed suspension with Aeromonas and Staphylococcus are presented in FIGURE 6.17. In pure culture (FIGURE 6.17 (a)), the coryneform attachment was lower at all 1/K values than the control value. This was also the case when the coryneform was in a mixed suspension with Aeromonas (FIGURE 6.17 (b)). There were, however, slight differences in the attachment of the coryneform in pure culture at the different 1/K values compared to the coryneform attachment in mixed suspension with Aeromonas. In mixed suspension with Staphylococcus (FIGURE 6.17 (c)), the attachment of the coryneform demonstrated marked differences compared to the results with the pure culture. The coryneform in mixed suspension did not attach in as greater numbers in the control as was seen with the pure culture. This resulted in the coryneform in mixed suspensions attaching to a greater extent when the 1/K value was 0.5 than the control. This was not the case for attachment of pure cultures of the coryneform where the coryneform attached to a lesser extent, when the 1/K value was 0.5, compared to the control. The importance of the salt present in the attachment solution during the attachment of the mixed suspension coryneform, again must be considered. This could be seen when a Na salt is present during the attachment of

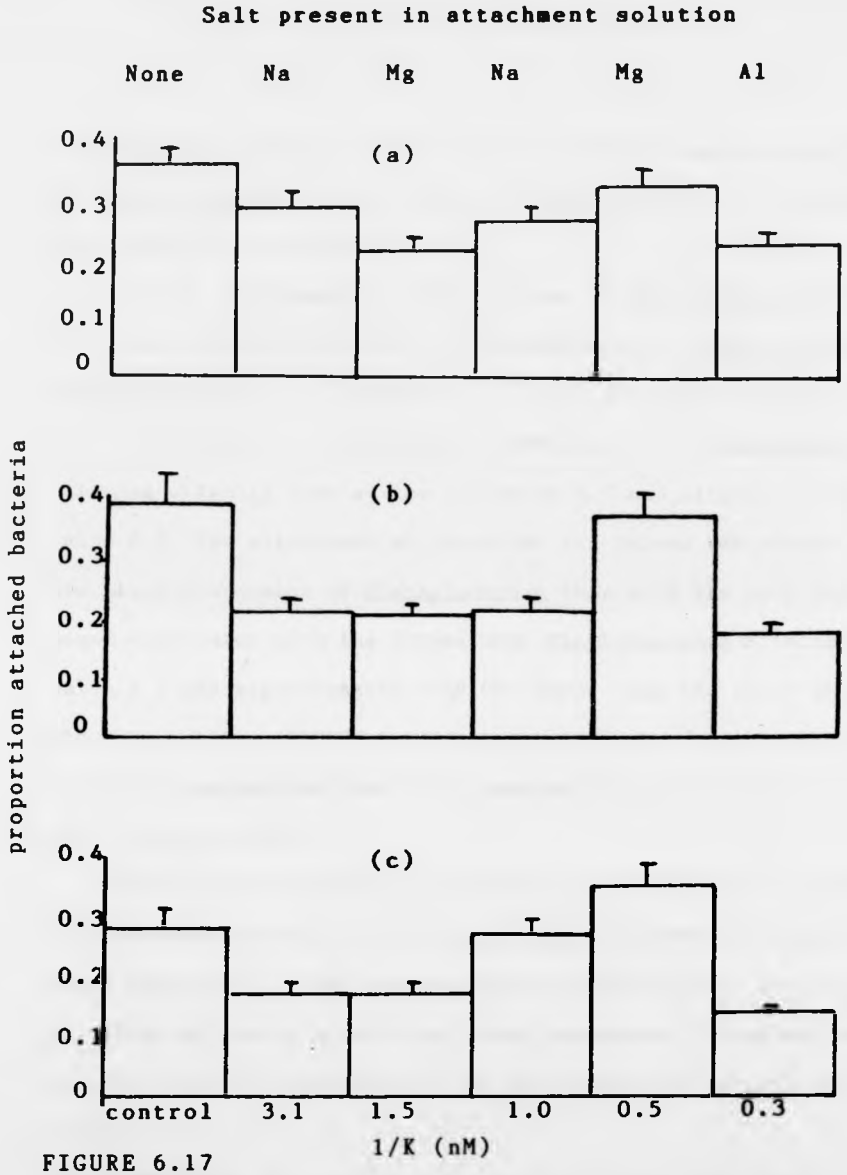


FIGURE 6.17
The effect of the electrical double-layer thickness on the attachment of (a) the coryneform in pure culture, (b) the coryneform in a mixed suspension with Aeromonas and (c) the coryneform in a mixed suspension with Staphylococcus.

N.B.- The bacteria were attached in solutions with different electrolyte types and concentrations. These solutions were incubated in a shaking incubator at 15°C.

The bacteria were first grown in batch culture in Medium 6 at 15°C. The bacteria were harvested and resuspended to produce the mixed suspensions required in the electrolyte solutions.

the coryneform in pure culture and in a mixed suspension with Aeromonas or Staphylococcus. In each case a different pattern of coryneform attachment was observed.

When the attachment of pure cultures of Staphylococcus (FIGURE 6.18 (a)) was investigated little difference between the attachment of Staphylococcus at 1/K values 3.1, 0.3 and the control was observed. In a mixed suspension with Aeromonas (FIGURE 6.18 (b)), Staphylococcus attached slightly less at the 1/K value 3.1 and slightly more at the 1/K value 0.3. The attachment at the other 1/K values was always lower with the mixed suspension of Staphylococcus than with the pure culture. In mixed suspension with the coryneform, Staphylococcus attachment at 1/K value 3.1 was significantly ($P>0.05$) lower than the value obtained with the pure culture (FIGURE 6.18 (c)). At 1/K value of 0.3, the attachment of Staphylococcus was similar in pure culture and in mixed suspension with the coryneform.

These results demonstrate that the 1/K, or the salt present in the attachment solution, could influence the attachment of a bacterium in a mixed suspension in the same way as the attachment of the pure cultures was affected. As with the other mixed suspension attachment results, the results obtained were dependent on the mixture of bacteria being investigated.

6.5 DISCUSSION

The results obtained in this Chapter suggest that the growth conditions influenced the attachment of a bacterium when it was in a mixed suspension, as they did when the bacterium was in a pure culture. These studies also suggest that a bacterium could influence the attachment of another bacterium to a surface when both were present in

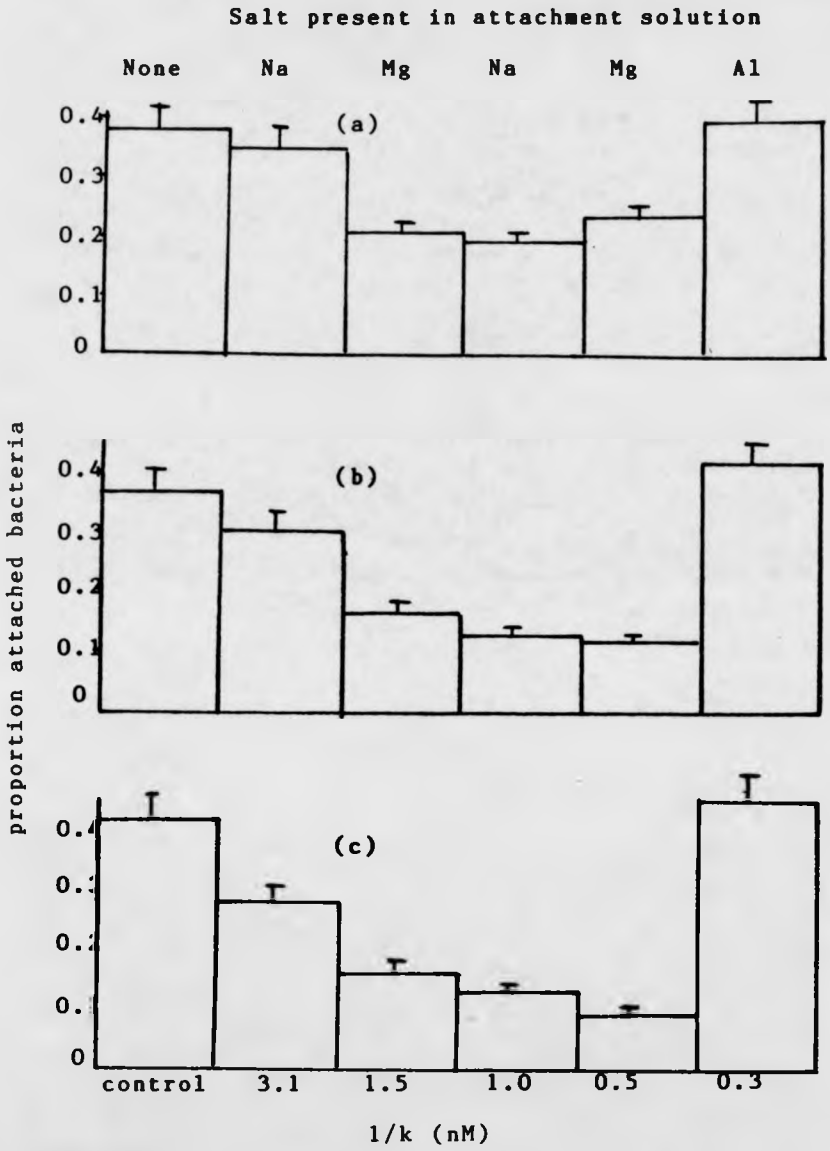


FIGURE 6.18
The effect of the electrical double-layer thickness on the attachment of (a) Staphylococcus in pure culture, (b) Staphylococcus in a mixed suspension with Aeromonas and (c) Staphylococcus in a mixed suspension with the coryneform.

N.B.- The bacteria were attached in solutions with different electrolyte types and concentrations. These solutions were incubated in a shaking incubator at 15°C.

The bacteria were first grown in batch culture in Medium 6 at 15°C. The bacteria were harvested and resuspended to produce the mixed suspensions required in the electrolyte solutions.

the attachment solution. There is no evidence in the literature to suggest that this cannot be the case, however, there is no direct evidence to support this claim. Rollinger (1987) has demonstrated that the presence of some species of bacteria could influence the presence or absence of other species on activated carbon. These indicator bacteria were thought to act as antagonists towards a range of other bacteria. These antagonists could contribute to nutritional competition, injury or chemical antibiosis. These results suggest that certain bacteria can greatly influence other bacteria when present in the same environment. Therefore, when considering the attachment of bacteria to surfaces it is not surprising that one species can influence the attachment of another when both are present in the same solution.

The methods used by bacteria to influence other species have been documented in the literature. The most common method is the production of chemicals by one bacterium to act on another. Means (1981) demonstrated that 22% of isolates from a chlorinated water system produced bacteriocin-like substances which inhibited the growth of other bacteria. In these experiments such substances if produced by a bacterium would not have influenced the growth of another bacterium as the bacteria were grown separately. However, it is possible that such substances could influence bacterial attachment when the bacteria were in mixed suspension. Other researchers have also demonstrated that substances produced by one species can influence the growth of another. For instance Psuedomonas can influence the growth of Haemophilus by the production of a bacteriocin (Morse, 1976) or a microcin (Asensio, 1976).

Further studies by Adams (1983) on attached Aeromonas suggested another method by which one bacterium could influence the attachment of

another. Aeromonas released an extracellular enzyme which could inactivate its own adhesin. It is possible that this enzyme could influence the attachment of other bacteria as well as Aeromonas and, therefore, influence the attachment of another bacterium. Indeed, such substances may be more relevant on the confined area of a surface than in the liquid phase where their effects would be expected to be minimal due to the dilution effect. This may be more important in the natural environment where the size of the liquid phase would dilute such a substance to insignificance.

Bacterial attachment has been studied in medical situations and these studies have provided information on the specificities of host-bacterial interactions. There is evidence from these studies that indigenous bacteria influence the colonisation of exogenous pathogens. Slots (1978) demonstrated that indigenous bacteria can serve as receptors for the adherence of pathogens. Reports have also indicated that pathogens can possess adhesions on their surfaces, which may be involved in specific interactions between different bacterial species (Komiyama, 1984). In the case where interbacterial adhesion occurred, the extent of adhesion of bacterial species was influenced by the species involved. This could in part account for some of the changes in bacterial attachment observed in these experiments where one bacterial species influences attachment of another bacterial species.

At present, it is impossible to say how one bacterium could influence the attachment of another to a surface. It is possible that different methods could be used by different bacteria. In some instances this change in attachment could be accidental, for example, due to waste products. On the other hand a more specific method may be involved.

CHAPTER SEVEN

ATTACHMENT OF PURE BACTERIAL SUSPENSIONS AND MIXED BACTERIAL SUSPENSIONS IN VIVO USING A MODEL SYSTEM

7.1 AIM

To investigate the effects of bacterial species, temperature and other environmental factors on attachment of bacterial species in a model system. These results are compared with those obtained for the laboratory-based studies reported in Chapters 4, 5 and 6.

7.2 INTRODUCTION

The results in Chapters 4, 5 and 6 have shown that the attachment of a bacterium to a solid surface can be influenced by another bacterium present in the liquid phase, or attached to the solid surface under laboratory conditions. Bacterial attachment was, therefore, investigated in Tocil Lake to establish if the changes in bacterial attachment observed in attachment experiments with cells grown in batch culture due to the presence of other species, would also occur in a natural aquatic environment. For this investigation, a model system was developed to study bacterial attachment under natural conditions in Tocil Lake, University of Warwick.

The importance of environmental factors, such as pH and temperature, on bacterial attachment was shown in Chapter 5. These same factors were again investigated to assess their importance to bacterial attachment under natural conditions. In these aquatic environments, other environmental factors could also be important in determining the attachment of bacteria to surfaces. These factors include salinity, particulate load (Bell, 1982; Yoon, 1990), turbulence (Eighmy, 1985) and

settling rates. (Ducklow, 1982). The importance of these factors in bacterial attachment was also considered in this chapter.

7.3 MATERIALS AND METHODS

7.3.1 Bacteria

Stock cultures of Aeromonas 2, Chromobacterium 2, the coryneform, Staphylococcus, Acinetobacter 1 and Pseudomonas 1 were set up in glucose mimimal media (Section 3.3.1). 10ml volumes of the stock cultures were centrifuged at 3,000g in a Beckman Centrifuge. The cells were washed in filtered lake water (0.2μ cellulose acetate filter), centrifuged and re-suspended in 5ml of the filtered lake water. The bacterial numbers in each of these bacterial preparations were determined using viable counts, (Section 3.3.1). This was to enable approximately equal numbers of the different species to be used in the model system. These bacterial preparations were stored at 4°C until used. This procedure did not appear to influence bacterial attachment if the storage time was below 10 days.

7.3.2 Model System

The model system (Figure 7.1) consisted of a 350ml glass vessel with 0.2μ cellulose acetate filters at each end. These filters were fitted to the system using Silcoset (Ambersil Ltd, Basingstoke). This Silcoset was found not to influence the viability, growth or attachment ability of the bacterial species used here (Appendix 3 Table 1.1).

The model system contained 14 glass surfaces of 16mm diameter. These surfaces were cleaned as in section 3.3.1. Once the system was sealed, it was sterilised by autoclaving at 120°C for 20 mins. The system was placed in the aquatic environment for 24hrs to fill with lake water. The system was quickly removed from the lake and inoculated with

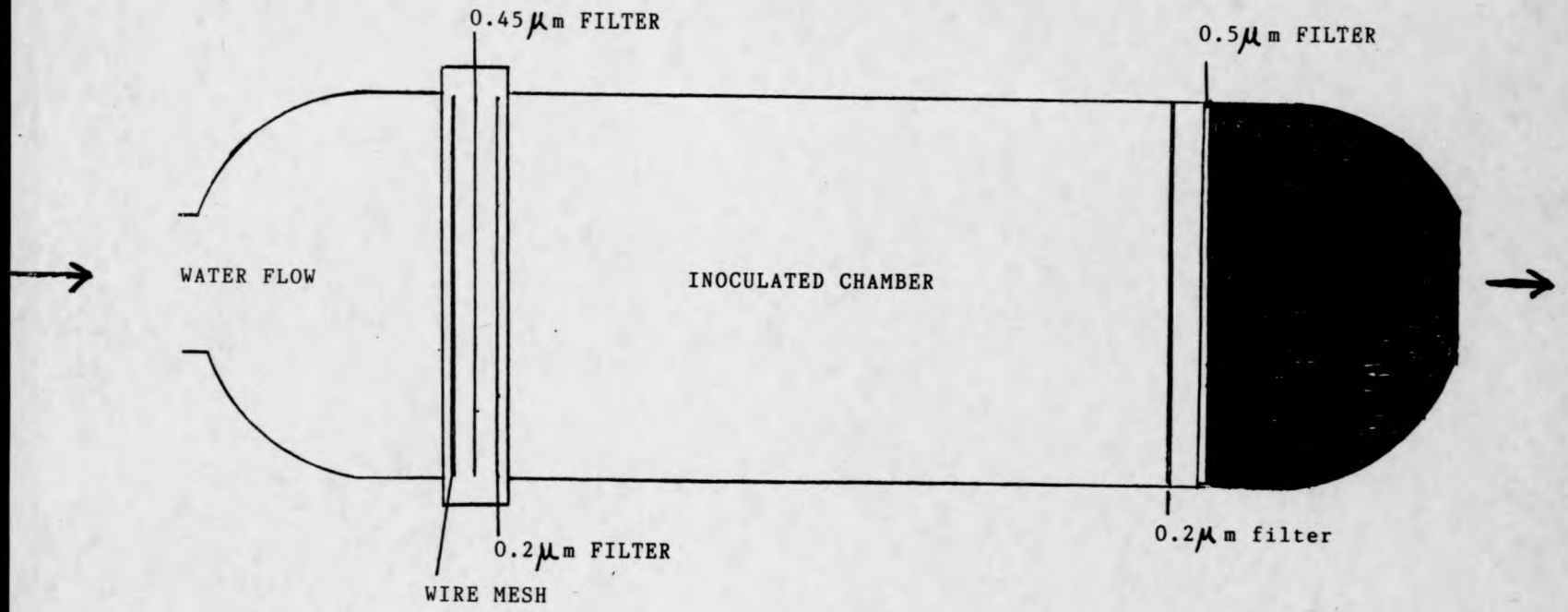


FIGURE 7.1 The model system in which attachment experiments were performed in. The model system was placed in Tocil Lake 24 hrs before the attachment experiments to fill up with lake water.

the bacterial suspension via the rubber bung. This was done in the field using a sterile syringe and needle, taking care to clean and flame the area of inoculation first, using a portable calor-gas stove. The surfaces were removed after 5 days and treated in the field as in Section 3.3.1. The bacterial population present in the model system after five days was determined by viable counts.

7.3.3 Experimental Conditions

The temperature of the aquatic environment was monitored twice a day at 0845 hrs and 1530 hrs using a field thermometer.

The pH of the aquatic environment was monitored daily by taking a 1L lake water sample and measuring the pH in the laboratory.

The organic and inorganic carbon levels before and after an experiment were determined in the laboratory using a Beckman Total Carbon analyser. The machine was standardised using organic and inorganic carbon solutiuons at 100mg C/l.

7.3.4 Immunofluorescence Microscopy

Each bacterial isolate, used in these experiments, had antibodies raised against it to be used in immunofluoresence microscopy. The bacterial cells were grown in nutrient broth in the presence of glass beads. These glass beads encouraged the growth of attached bacteria so that antibodies could be raised against the attachment organelles. The cells were harvested by centrifugation at 10,000g for 15 mins. The cells were washed five times in 0.1M phosphate buffer, pH 7.4, containing 0.3% (v/v) formaldehyde. The cells were resuspended and washed five times in 0.1M phosphate buffer, pH 7.4. The cell pellet was resuspended in 5ml of 0.1M phosphate buffer. 1ml of the bacterial suspension was then combined

with an equal volume of Freund incomplete adjuvant, (Difco Lab, Michigan), (Zambon,1984; Dahle,1982).

White rabbits were intravenously injected with the bacterial suspension + adjuvent mixture. After seven days another injection was given, followed by another after a further six weeks. Trial bleedings were then performed to determine the antibody titre using agglutination assays. (Finegold, 1978). Once the required titre was obtained the required quantity of rabbit blood was extracted. The rabbit blood was separated from the serum and the serum was heated to 56°C for three minutes to destroy the complement proteins. The antiserum was stored in small aliquots at -20°C until used.

The immunofluorescence assay used was described by Mouton (1978). A 10µl sample of a bacterial sample was placed on a glass coverslip, air dried and gently heat fixed. A checkerboard titration was then performed to determine the working titration for the antisera. A 10µl portion of twofold serial dilutions of the antisera in 0.1M phosphate buffer, pH 7.4, was placed on a heat-fixed bacterial sample for 20 mins. This sample was rinsed in phosphate buffer and washed in distilled water. The coverslips were incubated with 25µl of goat anti-rabbit immunoglobulin-G conjugated with fluorescein isothiocyanate serially diluted in phosphate buffer for 20 mins. The coverslips were rinsed and washed as before and mounted with glycerol in phosphate buffer (2:1 V/V).

The bacterial samples were examined using a microscope equipped for phase-contrast illumination and incident-light fluorescence. The working titre required for each antisera were determined by the titre which gave good fluorescence with good cell envelope definition. The surfaces obtained from the model system were heat fixed and investigated using

this immunofluorescence assay and the antisera working titre outlined above.

7.4 RESULTS

Appendix FIGURE 3 (a) and (b) gives examples of the types of biofilms obtained during the attachment experiments in this chapter.

7.4.1 Pure Culture vrs Mixed Suspension Attachment

The bacterial population present in the model system, after each attachment experiment with mixed suspensions, consisted of varying proportions of the original species inoculated into the sytem. In every case, each of the species inoculated into the model system were isolated from the bacterial population present in the model system at the end of the attachment experiments.

7.4.1.(A) Two-membered suspensions

Table 7.1 shows the results obtained for the attachment of pure cultures plus two-membered mixtures of these bacteria in the model system suspended in Tocil Lake water.

In pure culture, the best attachment was seen with cultures of Staphylococcus (overall mean 0.6275) and Aeromonas (0.5423) whilst Acinetobacter did not attach at all.

In the mixed suspensions, Acinetobacter still did not attach to the glass surfaces and this organism always had an inhibitory effect on the other members of the mixed community reducing attachment by at least 50% for the other species. Acinetobacter possibly produces an inhibitory substance which coats the surface preventing other organisms forming a biofilm.

Staphylococcus attachment was also much reduced in the prescence of other bacteria, reducing the mean from 0.6275 to 0.0992, with a range of

TABLE 7.1 Attachment of bacteria in pure culture and in a mixed suspension with one other bacterial species in the model system. The temperatures during these experiments were within the range 8°C to 15°C.

BACTERIA INVESTIGATED		ATTACHMENT RESULTS PURE CULTURE	MIXED SUSPENSION
Aeromonas	+	0.5327 (0.0981)	0.4261 (0.0827)
Chromobacterium		0.3116 (0.0647)	0.1378 (0.0295)
Aeromonas	+	0.5780 (0.1138)	0.4183 (0.0940)
coryneform		0.4729 (0.0906)	0.0874 (0.0176)
Aeromonas	+	0.5221 (0.0988)	0.6825 (0.1256)
Staphylococcus		0.6824 (0.1532)	0.0613 (0.0102)
Aeromonas	+	0.5620 (0.0953)	0.3051 (0.0590)
Acinetobacter		0.0000	0.0000
Aeromonas	+	0.5167 (0.0904)	0.3528 (0.0716)
Pseudomonas		0.3475 (0.0827)	0.1158 (0.0238)
Chromobacterium	+	0.3381 (0.0736)	0.3150 (0.0720)
coryneform		0.4693 (0.0893)	0.0568 (0.0105)
Chromobacterium	+	0.2996 (0.0724)	0.4236 (0.0801)
Staphylococcus		0.5936 (0.0124)	0.0420 (0.0082)
Chromobacterium	+	0.3614 (0.0716)	0.1938 (0.0410)
Acinetobacter		0.0000	0.0000
Chromobacterium	+	0.3251 (0.0695)	0.3526 (0.0658)
Pseudomonas		0.3427 (0.0671)	0.2316 (0.0491)
coryneform	+	0.5320 (0.0978)	0.5147 (0.0892)
Staphylococcus		0.6198 (0.1152)	0.1268 (0.0205)
coryneform	+	0.4470 (0.0783)	0.2130 (0.0396)
Acinetobacter		0.0000	0.0000
coryneform	+	0.4946 (0.0790)	0.0615 (0.0099)
Pseudomonas		0.3520 (0.0743)	0.2841 (0.0517)
Staphylococcus	+	0.6370 (0.1218)	0.1938 (0.0391)
Acinetobacter		0.0000	0.0000
Staphylococcus	+	0.6046 (0.1137)	0.0721 (0.0116)
Pseudomonas		0.3271 (0.0645)	0.3016 (0.0650)
Pseudomonas	+	0.3148 (0.0598)	0.0985 (0.0192)
Acinetobacter		0.0000	0.0000

N.B- Pure culture attachments were performed with each mixed suspension to monitor any change in a bacterium's attachment ability.

0.0420 to 0.1938 compared to 0.5936 to 0.6824 for the pure culture. This is a highly significant decrease in attachment potential for this organism and presumably occurs because of interactions between the bacteria concerned, or because the surface attachment sites become occupied by the competitor rather than by Staphylococcus. In fact, the highest attachment occurred in the prescence of Acinetobacter, the one organism which did not attach at all.

Aeromonas attachment was reduced slightly but not significantly from a mean of 0.5423 to 0.4370, but the range of values increased with attachment being significantly higher in the prescene of Staphylococcus and lower in the presence of Acinetobacter. With the other species, there was a slight but not significant decrease in attachment.

The attachment of the coryneform was greatly inhibited by Aeromonas, Chromobacterium and Pseudomonas, slightly reduced by Acinetobacter, but unaffected by Staphylococcus. There was a much wider range of attachment values from 0.0568 to 0.5147 for this organism in mixed culture than in pure culture (0.4470 to 0.5320).

The attachment of Chromobacterium was reduced in the presence of Aeromonas and Acinetobacter, enhanced by Staphylococcus but unaffected by Pseudomonas and by the coryneform.

The attachment of Pseudomonas was only greatly affected by Acinetobacter and Aeromonas which were strongly inhibitory. The other species did not affect Pseudomonas attachment significantly.

7.4.1.(B) Three-membered suspensions

The results obtained when three bacterial species (Table 7.2) were used in a mixed suspension again indicated that the attachment of a bacterium could be influenced by the presence of other species.

TABLE 7.2 Attachment of bacteria in pure culture and in a mixed suspension with two other bacterial species in the model system. The temperatures during these experiments were within the range 8°C to 15°C.

BACTERIA INVESTIGATED		ATTACHMENT RESULTS PURE CULTURE	MIXED SUSPENSION
Aeromonas	+	0.4871 (0.0891)	0.4295 (0.0860)
Chromobacterium	+	0.3095 (0.0618)	0.0810 (0.0114)
coryneform		0.5248 (0.0921)	0.0251 (0.0056)
Aeromonas	+	0.5283 (0.0865)	0.7143 (0.1106)
Chromobacterium	+	0.3614 (0.0628)	0.3217 (0.0592)
Staphylococcus		0.6490 (0.0986)	0.0531 (0.0092)
Aeromonas	+	0.5174 (0.0882)	0.2875 (0.0478)
Chromobacterium	+	0.3468 (0.0610)	0.1144 (0.0296)
Acinetobacter		0.0000	0.0000
Aeromonas	+	0.5036 (0.0923)	0.4750 (0.0885)
Chromobacterium	+	0.3286 (0.0579)	0.1739 (0.0341)
Pseudomonas		0.2958 (0.0472)	0.0937 (0.0173)
Aeromonas	+	0.4876 (0.0853)	0.6134 (0.0968)
coryneform	+	0.4931 (0.0882)	0.1140 (0.0206)
Staphylococcus		0.6205 (0.0921)	0.0413 (0.0071)
Aeromonas	+	0.4627 (0.0813)	0.2874 (0.0451)
coryneform	+	0.5231 (0.0952)	0.0512 (0.0085)
Acinetobacter		0.0000	0.0000
Aeromonas	+	0.5081 (0.0830)	0.3827 (0.0562)
coryneform	+	0.5052 (0.0792)	0.0415 (0.0076)
Pseudomonas		0.3264 (0.0591)	0.1436 (0.0269)
Aeromonas	+	0.5032 (0.0871)	0.3728 (0.0557)
Staphylococcus	+	0.6247 (0.0994)	0.0327 (0.0082)
Acinetobacter		0.0000	0.0000
Aeromonas	+	0.4861 (0.0782)	0.6481 (0.1147)
Staphylococcus	+	0.6652 (0.0958)	0.0319 (0.0058)
Pseudomonas		0.3167 (0.0530)	0.1538 (0.0286)
Aeromonas	+	0.4804 (0.0632)	0.2169 (0.0431)
Pseudomonas	+	0.3217 (0.0581)	0.0926 (0.0158)
Acinetobacter		0.0000	0.0000
Chromobacterium	+	0.3280 (0.0593)	0.3674 (0.0610)
coryneform	+	0.4845 (0.0698)	0.0562 (0.0092)
Staphylococcus		0.6310 (0.0952)	0.0428 (0.0078)

N.B- Pure culture attachments were performed with each mixed suspension to monitor any change in a bacterium's attachment ability.

TABLE 7.2 (Continued)

BACTERIA INVESTIGATED	ATTACHMENT RESULTS PURE CULTURE	MIXED SUSPENSION
Chromobacterium + coryneform + Acinetobacter	0.3325 (0.0564) 0.4915 (0.0650) 0.0000	0.1576 (0.0218) 0.0351 (0.0067) 0.0000
Chromobacterium + coryneform + Pseudomonas	0.3056 (0.0520) 0.4685 (0.0668) 0.3152 (0.0553)	0.2874 (0.0392) 0.0479 (0.0096) 0.1469 (0.0280)
Chromobacterium + Staphylococcus + Acinetobacter	0.2970 (0.0417) 0.6741 (0.1148) 0.0000	0.1258 (0.0180) 0.0316 (0.0074) 0.0000
Chromobacterium + Staphylococcus + Pseudomonas	0.2861 (0.0382) 0.6137 (0.0970) 0.3253 (0.0440)	0.3469 (0.0425) 0.0521 (0.0092) 0.2718 (0.0372)
Chromobacterium + Pseudomonas + Acinetobacter	0.2938 (0.0375) 0.3127 (0.0392) 0.0000	0.1587 (0.0254) 0.1035 (0.0198) 0.0000
coryneform + Staphylococcus + Acinetobacter	0.4482 (0.0664) 0.6631 (0.0968) 0.0000	0.2538 (0.0347) 0.1170 (0.0201) 0.0000
coryneform + Staphylococcus + Pseudomonas	0.4528 (0.0630) 0.6015 (0.0899) 0.3053 (0.0392)	0.0231 (0.0062) 0.0712 (0.0126) 0.4627 (0.0637)
coryneform + Pseudomonas + Acinetobacter	0.4630 (0.0629) 0.3185 (0.0386) 0.0000	0.0347 (0.0072) 0.1659 (0.0317) 0.0000
Staphylococcus + Pseudomonas + Acinetobacter	0.6052 (0.0938) 0.2846 (0.0362) 0.0000	0.0814 (0.0115) 0.1257 (0.0261) 0.0000

N.B- Pure culture attachments were performed with each mixed suspension to monitor any change in a bacterium's attachment ability.

Aeromonas was always the dominant member of the biofilm attaching least as well as in pure culture, in the presence of the other bacteria, except for Acinetobacter which significantly reduced the attachment of Aeromonas compared to the attachment of pure cultures of this organism.

Chromobacterium and Pseudomonas rarely attached as well in mixed culture as they did in pure culture, although there were exceptional cases, Chromobacterium plus Pseudomonas plus Staphylococcus where the presence of Staphylococcus increased the attachment of Chromobacterium. There were other cases where the presence of a Gram-positive bacterium increased the attachment of one or both of Chromobacterium and Pseudomonas.

Staphylococcus never attached as well in mixed culture as it did in pure culture, with the attachment value decreasing some 90% usually. The presence of Staphylococcus also greatly affected the attachment of other species usually increasing the attachment of one of the members of the three membered community, but there were cases where Staphylococcus appeared to be inhibitory to attachment. It is assumed that the inhibition of attachment of Staphylococcus was caused by the competition for attachment sites being won by the other species which were usually Gram-negative.

The coryneform was usually worse at attaching in the presence of competitors than in pure culture. Acinetobacter, however, never attached in pure or mixed culture. Indeed, in mixed culture Acinetobacter seemed to inhibit the attachment of the other members of the community. Even Aeromonas which seemed to be unaffected by other species showed some decrease in attachment ability in the presence of Acinetobacter.

7.4.1.(C) Four-membered suspensions

Table 7.3 shows the result for four membered-suspensions compared to the pure culture suspensions in the model system in Tocil Lake water.

The results are more complex than in the previous simpler systems but once again, where it was one of the community members, Aeromonas attached better than the other bacteria in the suspension, although as expected the best attachment in pure culture was shown by Staphylococcus. Once again Acinetobacter failed to attach and reduced the attachment ability of other species. In these complex suspensions, none of the other species attached at any significant rate and a 90% reduction in attachment ability was commonplace. Even the attachment of Aeromonas was always reduced compared to the attachment ability of the pure culture (the overall mean was significantly decreased from 0.5052 to 0.2658. The coryneform also failed to attach in three of the six combinations and attachment was very much reduced in the other three cases (overall attachment 0.0364 compared to 0.4288 for the pure culture). As with the other suspensions, Staphylococcus attached poorly in the mixed suspension (overall attachment 0.0328 compared to 0.6126 for the pure culture).

7.4.1.(D) Five-membered suspensions

Table 7.4 shows the results for the pure cultures and five-membered suspensions of bacteria in the model system in Tocil Lake water.

Again, only Aeromonas attached to any great extent but attachment was always lower than for pure culture. All the other species attached significantly worse than when in pure culture, with Acinetobacter again failing to attach at all.

TABLE 7.3 Attachment of bacteria in pure culture and in a mixed suspension with three other bacterial species in the model system. The temperatures during these experiments were within the range 8°C to 15°C.

BACTERIA INVESTIGATED		ATTACHMENT RESULTS PURE CULTURE	MIXED SUSPENSION
Aeromonas	+	0.4872 (0.0681)	0.3875 (0.0560)
Chromobacterium	+	0.2973 (0.0448)	0.0759 (0.0106)
coryneform	+	0.4530 (0.0612)	0.0000
Staphylococcus		0.6259 (0.0926)	0.0000
Aeromonas	+	0.5038 (0.0675)	0.2552 (0.0389)
Chromobacterium	+	0.3255 (0.0420)	0.0651 (0.0092)
coryneform	+	0.4297 (0.0614)	0.0000
Acinetobacter		0.0000	0.0000
Aeromonas	+	0.5276 (0.0685)	0.3629 (0.0520)
Chromobacterium	+	0.3185 (0.0431)	0.0682 (0.0089)
coryneform	+	0.4075 (0.0534)	0.0000
Pseudomonas		0.2863 (0.0372)	0.0429 (0.0061)
Aeromonas	+	0.4995 (0.0623)	0.1947 (0.0286)
Chromobacterium	+	0.3136 (0.0428)	0.0368 (0.0050)
Staphylococcus	+	0.6057 (0.0896)	0.0000
Acinetobater		0.0000	0.0000
Aeromonas	+	0.5126 (0.0665)	0.1438 (0.0237)
Chromobacterium	+	0.2874 (0.0380)	0.0421 (0.0059)
Pseudomonas	+	0.2910 (0.0405)	0.0472 (0.0065)
Acinetobacter		0.0000	0.0000
Aeromonas	+	0.4837 (0.0624)	0.3527 (0.0531)
Chromobacterium	+	0.2916 (0.0436)	0.0586 (0.0076)
Pseudomonas	+	0.3021 (0.0435)	0.0382 (0.0058)
Staphylococcus		0.6170 (0.0950)	0.0000
Aeromonas	+	0.5219 (0.0652)	0.1635 (0.0281)
coryneform	+	0.4178 (0.0550)	0.0347 (0.0054)
Staphylococcus	+	0.5992 (0.0881)	0.0326 (0.0047)
Acinetobacter		0.0000	0.0000
Aeromonas	+	0.4615 (0.0620)	0.3516 (0.0492)
coryneform	+	0.4327 (0.0597)	0.0000
Staphylococcus	+	0.6210 (0.0851)	0.0000
Pseudomonas		0.2915 (0.0356)	0.0512 (0.0085)

N.B- Pure culture attachments were performed with each mixed suspension to monitor any change in a bacterium's attachment ability.

TABLE 7.3 (Continued)

BACTERIA INVESTIGATED		ATTACHMENT RESULTS PURE CULTURE	MIXED SUSPENSION
Aeromonas	+	0.4526 (0.0627)	0.1726 (0.0225)
coryneform	+	0.4648 (0.0581)	0.0000
Acinetobacter	+	0.0000	0.0000
Pseudomonas		0.3016 (0.0413)	0.0516 (0.0087)
Aeromonas	+	0.4627 (0.0659)	0.1857 (0.0238)
Staphylococcus	+	0.6118 (0.0815)	0.0000
Acinetobacter	+	0.0000	0.0000
Peudomonas		0.2950 (0.0340)	0.0348 (0.0051)
Chromobacterium	+	0.3169 (0.0440)	0.2537 (0.0365)
coryneform	+	0.4328 (0.0539)	0.0426 (0.0061)
Staphylococcus	+	0.6150 (0.0921)	0.0330 (0.0046)
Pseudomonas		0.3158 (0.0416)	0.0492 (0.0060)
Chromobacterium	+	0.3259 (0.0452)	0.1362 (0.0253)
coryneform	+	0.4317 (0.0528)	0.0319 (0.0045)
Pseudomonas	+	0.2861 (0.0427)	0.0353 (0.0049)
Acinetobacter		0.0000	0.0000
Chromobacterium	+	0.3258 (0.0417)	0.1420 (0.0217)
coryneform	+	0.4581 (0.0576)	0.0329 (0.0059)
Staphylococcus	+	0.6036 (0.0847)	0.0361 (0.0054)
Acinetobacter		0.0000	0.0000
Chromobacterium	+	0.3360 (0.0426)	0.1295 (0.0224)
Staphylococcus	+	0.6415 (0.0905)	0.0000
Acinetobacter	+	0.0000	0.0000
Pseudomonas		0.2985 (0.0362)	0.0391 (0.0056)
coryneform	+	0.4328 (0.0583)	0.0368 (0.0051)
Staphylococcus	+	0.6159 (0.0892)	0.0000
Acinetobacter	+	0.0000	0.0000
Pseudomonas		0.3150 (0.0398)	0.1279 (0.0195)

N.B- Pure culture attachments were performed with each mixed suspension to monitor any changes in a bacterium's attachment ability.

TABLE 7.4 Attachment of bacteria in pure culture and in a mixed suspension with four other bacterial species in the model system. The temperatures during these experiments were within the range 8°C to 15°C.

BACTERIA INVESTIGATED		ATTACHMENT RESULTS PURE CULTURE	MIXED SUSPENSION
Aeromonas	+	0.4915 (0.0547)	0.3872 (0.0490)
Chromobacterium	+	0.2836 (0.0361)	0.0421 (0.0065)
Staphylococcus	+	0.6315 (0.0987)	0.0368 (0.0049)
coryneform	+	0.4835 (0.0558)	0.0320 (0.0042)
Pseudomonas		0.2960 (0.0375)	0.0624 (0.0092)
Aeromonas	+	0.4681 (0.0524)	0.1369 (0.0236)
Chromobacterium	+	0.3154 (0.0445)	0.0316 (0.0034)
coryneform	+	0.4620 (0.0548)	0.0427 (0.0061)
Pseudomonas	+	0.2962 (0.0416)	0.0530 (0.0073)
Acinetobacter		0.0000	0.0000
Aeromonas	+	0.5120 (0.0562)	0.2260 (0.0326)
Chromobacterium	+	0.2916 (0.0369)	0.0527 (0.0076)
coryneform	+	0.4428 (0.0510)	0.0371 (0.0045)
Staphylococcus	+	0.6120 (0.0875)	0.0291 (0.0040)
Acinetobacter		0.0000	0.0000
Aeromonas	+	0.4840 (0.0531)	0.1528 (0.0216)
Chromobacterium	+	0.3016 (0.0397)	0.0513 (0.0076)
Staphylococcus	+	0.6321 (0.0926)	0.0371 (0.0052)
Acinetobacter	+	0.0000	0.0000
Pseudomonas		0.3050 (0.0359)	0.0518 (0.0070)
Aeromonas	+	0.4892 (0.0528)	0.1526 (0.0219)
coryneform	+	0.4730 (0.0551)	0.0327 (0.0050)
Staphylococcus	+	0.6386 (0.0912)	0.0380 (0.0057)
Acinetobacter	+	0.0000	0.0000
Pseudomonas		0.2952 (0.0360)	0.0613 (0.0088)
Chromobacterium	+	0.3149 (0.0406)	0.2370 (0.0328)
coryneform	+	0.4510 (0.0561)	0.0368 (0.0047)
Pseudomonas	+	0.3182 (0.0419)	0.0627 (0.0093)
Staphylococcus	+	0.6062 (0.0895)	0.0237 (0.0036)
Acinetobacter		0.0000	0.0000

N.B- Pure culture attachments were performedwith each mixed suspension to monitor any change in a bacterium's attachment ability.

7.4.1. (E) Six-membered suspension

In this single experiment with all six members of the community together, again only Aeromonas attached but this was significantly reduced compared to the pure culture. Both Staphylococcus and Acinetobacter failed to attach and Chromobacterium, Pseudomonas and the coryneform attachment abilities were significantly reduced (Table 7.5).

7.4.2 Temperature and Attachment

The above experiments were performed when the average temperature of the lake was 9°C, during the winter months the average temperature of the lake was 3°C. This allowed the model system and the attachment abilities of the bacteria to be investigated at a different temperature range. The results for the attachment of a selection of species in pure culture and in mixed suspensions at an average temperature of 3°C are shown in Table 7.6. Only a small selection of the mixed suspensions used at 9°C could be investigated at 3°C due to the lake water temperature not falling into the 3°C range for any lengh of time throughout the year.

There was no significant ($P>0.05$) difference in the attachment of pure cultures of Aeromonas or of Chromobacterium at 3°C or 9°C. The experimental temperatures used in these attachment experiments were lower than those used in the attachment experiments for batch cultures (Chapter 4). The results obtained, however, were similar with no significant change in the attachment of Aeromonas or Chromobacterium in pure culture demonstrated when the attachment temperature was increased from 15°C to 25°C. The attachment of pure cultures of Staphylococcus and the coryneform in the model system was significantly reduced at 3°C compared to 9°C. These model system results could not be compared with

TABLE 7.5 Attachment of bacteria in pure culture and in a mixed suspension with five other bacterial species in the model system. The temperatures during these experiments were within the range 8°C to 15°C.

BACTERIA INVESTIGATED		ATTACHMENT RESULTS PURE CULTURE	MIXED SUSPENSION
Aeromonas	+	0.5106 (0.0627)	0.1572 (0.0237)
Chromobacterium	+	0.2931 (0.0382)	0.0416 (0.0053)
Staphylococcus	+	0.6352 (0.0917)	0.0000
coryneform	+	0.4826 (0.0570)	0.0319 (0.0043)
Acinetobacter	+	0.0000	0.0000
Pseudomonas		0.3142 (0.0408)	0.0396 (0.0050)

N.B- Pure culture attachments were performed with each mixed suspension to monitor any change in a bacterium's attachment ability.

TABLE 7.6 Attachment of bacteria in pure culture and in a mixed suspension with different numbers of other bacterial species in the model system. The temperature during these experiments were within the range 1°C to 5°C.

BACTERIA INVESTIGATED		ATTACHMENT RESULTS PURE CULTURE	MIXED SUSPENSION
Aeromonas	+	0.5626 (0.0630)	0.4362 (0.0560)
Chromobacterium		0.4872 (0.0528)	0.2731 (0.0336)
Aeromonas	+	0.5482 (0.0605)	0.4382 (0.0508)
Acinetobacter		0.0000	0.0000
Aeromonas	+	0.5379 (0.0616)	0.5270 (0.0642)
Staphylococcus		0.0000	0.0000
Chromobacterium	+	0.3827 (0.0431)	0.2158 (0.0296)
Acinetobacter		0.0000	0.0000
Chromobacterium	+	0.3650 (0.0415)	0.3728 (0.0448)
Staphylococcus		0.0000	0.0000
coryneform	+	0.2875 (0.0361)	0.2653 (0.0358)
Staphylococcus		0.0000	0.0000
Aeromonas	+	0.5372 (0.0617)	0.3921 (0.0473)
Chromobacterium	+	0.3659 (0.0435)	0.0825 (0.0094)
Acinetobacter		0.0000	0.0000
Aeromonas	+	0.5421 (0.0630)	0.4316 (0.0524)
Chromobacterium	+	0.3487 (0.0451)	0.1734 (0.0247)
Staphylococcus		0.0000	0.0000
coryneform	+	0.2951 (0.0328)	0.1490 (0.0226)
Staphylococcus	+	0.0000	0.0000
Acinetobacter		0.0000	0.0000
Aeromonas	+	0.5236 (0.0618)	0.3729 (0.0460)
Chromobacterium	+	0.3519 (0.0421)	0.0695 (0.0089)
Staphylococcus	+	0.0000	0.0000
Acinetobacter		0.0000	0.0000
Aeromonas	+	0.5128 (0.0623)	0.4620 (0.0539)
Chromobacterium	+	0.3268 (0.0462)	0.1327 (0.0241)
coryneform	+	0.2731 (0.0360)	0.0426 (0.0057)
Staphylococcus		0.0000	0.0000

N.B- Pure culture attachments were performed with each mixed suspension to monitor any change in a bacterium's attachment ability. Only a selection of the mixed suspensions investigated at the other temperature range were studied here due to the limited period of time the lake water was at this temperature range.

batch culture results as no change in the attachment of the Staphylococcus or the coryneform was observed when the attachment temperature was increased from 15°C to 25°C.

In mixed suspensions at 3°C there was a significant reduction in the attachment of Aeromonas compared to the pure culture results , as there was in most cases at 9°C. The attachment of the Chromobacterium was also significaly reduced in mixed suspensions (with the exception of the mixed suspension with Staphylococcus) again this was also seen at 9°C. The coryneform was also affected by the other members of the mixed suspensions as at 9°C. Staphylococcus had no effect on coryneform attachment but the other members including Acinetobacter did reduce coryneform attachment. The effects of Acinetobacter on attachment of other species was slightly less at 3°C than it was at 9°C.

7.4.3 Bacterial Biofilms

The bacterial biofilms obtained in these studies consisted of bacterial cells densely packed together to form the biofilm. The bacterial cells themselves were generally smaller than those which attached in batch cultures. In some cases, these bacterial cells were one tenth the size of the bacterial cells obtained in batch culture experiments. The only species which could be microscopically identified was Staphylococcus. This could be done if the attached bacteria were Gram stained and this resulted in the Gram-positive Staphylococcus being identified. The other species in the biofilm appeared similar and could not be distinguished.

On microscopic investigation of the biofilm, Staphylococcus was not identified in biofilms formed from mixed suspensions, even though it was present in the biofilm. Staphylococcus must, therefore, have been

covered by the other species in the biofilm. This suggests that Staphylococcus in a mixed bacterial suspension was one of the first species to attach to the surface. To try and establish where the other species were sited in the biofilms, immunofluorescence techniques were used. It was hoped that the bacteria on top of the biofilm could be identified using antisera linked to a fluorescent stain raised against each bacterium.

7.4.4 Immunofluorescence Studies

Antisera were raised against Chromobacterium, Pseudomonas and coryneform. For an unknown reason an antiserum could not be raised against Aeromonas. When these antisera were used to investigate the bacterial biofilms, no immunofluorescence was observed in the biofilm. The antisera worked on the dried smears of the original bacterial isolates. Therefore, the attached bacteria must have changed sufficiently for the antisera not to identify them.

7.5 DISCUSSION

7.5.1 Biofilm and its Environment

The bacterial attachment observed in different aquatic environments can vary greatly depending on the aquatic environment being investigated (Goulder, 1977; Hanson, 1977). Therefore, the biofilms obtained in batch experiments and in the model system would be expected to be different. Environmental factors such as seasonal changes within Tocil lake, could influence bacterial attachment in the model system as was demonstrated with the temperature experiments. Unfortunately very few studies on the factors influencing bacterial attachment in aquatic environments have been carried out (Cammen, 1982), and the information available for the attachment of bacteria to surfaces in aquatic

environments is limited. Due to this shortage of information on bacterial attachment in natural aquatic environments only suggestions can be made of the environmental factors which could influence bacterial attachment.

The biofilms obtained with the model system were more dense in the numbers of bacteria involved. As this biofilm develops, metabolic problems would occur, the main one being the availability of oxygen within the biofilm. This would not be a important factor in laboratory studies due to the short attachment times involved. When this problem is studied with a pure culture, during colony formation, oxygen can become limited in certain areas of the developing colony leading to the development of anoxic areas. As these anoxic areas increase, toxic metabolite accumulation and eventual cessation of growth can occur (Fraleigh, 1986). If this problem occurs with pure cultures, then it should also occur within mixed bacterial biofilms. A build up of toxic metabolites or a lack of oxygen within a biofilm would influence its development. Therefore, these factors could be important in biofilm development in aquatic environments, especially if the biofilm becomes too extensive. This might be the case in natural aquatic environments, although numerous other factors could be more important in determining the size of the resulting biofilm.

Characteristics of free-floating and attached bacteria of 44 diverse freshwater and seawater environments were studied to determine the factors which significantly influence bacterial attachment (Bell, 1982). The factors found to be of significance were salinity, particulate load and the heterotrophic uptake of glucose and amino-acids. Salinity and particulate load were not thought to be important factors in these

experiments. The salinity of the lake investigated was shown to change very little during the experimental periods. Also the particulate matter within the model system changed very little due to the filters on the model system. It was thought that the uptake of nutrients by the different species present would have more influence on the resulting biofilm. It can be seen from the results in Chapter 4 that nutrients have an important influence on biofilm development.

With phytoplankton (Albright, 1986) and possibly with some bacteria, senescent members of the populations could release nutrients which could be used by the bacteria in close proximity to these microorganisms. This could lead to bacteria attaching in increasing numbers in these areas, and in some cases, large aggregates of bacteria have been seen to form. On investigation the major component holding these aggregates together is the bacterial glycocalyx (Biddanda, 1985). Usually this glycocalyx formed only in bacteria in nutrient-rich conditions. Therefore, the nutrient conditions which allowed this glycocalyx to form were created due to the senescent members of the populations involved. This particular situation may not be common in natural aquatic environments, but the importance of the different nutrient sources available to bacteria and their influence on bacterial attachment must be considered. A sudden change in the main nutrient in an aquatic environment could result in a completely new biofilm forming on a solid surface within that environment (Chapter 5).

Apart from the environmental conditions already investigated in the laboratory studies, other environmental factors found in natural aquatic environments may influence the biofilms found on surfaces in these aquatic environments. Eighmy (1985), has demonstrated that turbulence

can be an important factor to consider in natural aquatic environments, as turbulence has been shown to influence the nutrient transport systems of bacteria in biofilms. These transport systems were affected as turbulence caused changes in the outer membranes, the site of these transport systems of the bacteria investigated. In these experiments bacteria were shown to have alternative transport systems which could be used when the bacterium adapted to the turbulent conditions. If this occurred in Tocil Lake where turbulence varied depending on factors such as rainfall in the areas of Tocil Lake where the model systems were situated, this would result in different bacterial transport systems being used. If the bacteria involved in a biofilm did not have an alternative transport system then the bacterial biofilm could change altogether depending on the bacteria best suited to the turbulent conditions.

In natural aquatic environments, another factor which could affect the bacterial population and, therefore, the attached bacteria, is the phenonemon of settling out. Free-living bacteria have lower rates of sinking than that of attached bacteria. In some cases up to 67% of the bacterial population of an aquatic system has been seen to settle out (Ducklow, 1982). The importance of this process in aquatic environments will depend on the bacteria which are settling out and on the importance of the attached bacteria within the aquatic system. If in conditions of high particulate load within a system, the dominant attached bacteria could be lost from the liquid phase due to settling out, this would result in future biofilms having a new dominant bacterium. Attached bacteria have also demonstrated a seasonal trend in activity (Lovell, 1985), with late summer being the time of year when attached bacterial

activity was at its greatest. Therefore, attached bacteria in summer months may be influenced more by nutrient availability than any other factor, due to their greater activity. This could indicate that the importance of other parameters on attached bacteria will depend on the season in which the investigation was carried out.

7.5.2 Microorganisms in Aquatic Environments

Bacteria are not the only organisms present in natural aquatic environments. It is, therefore, possible that the other organisms present will influence how a biofilm develops. In succession studies, this has been indicated when different bacteria replace one another in the biofilm development (Chapter 1). Also in the latter stages of succession, organisms such as protozoa influence the biofilm development and, therefore, must be studied (Fletcher, 1982). When the different organisms are studied in more detail some ideas on how they could influence bacterial biofilms are obtained

The importance of bacteria on the attachment of other bacteria has been discussed in Chapter 6. A good example of this is when biologically activated carbon (BAC) was investigated in filters at different water plants, specialised bacterial communities developed. Species such as Pseudomonas. Flavobacterium and Acinetobacter. were the dominant bacteria on the BAC. Hygienically-relevant bacterial species such as coliforms were found in the effluent of these plants but never on the BAC (Rollinger, 1987). As discussed in Chapter 6 the bacteria were thought to act as antagonists towards coliforms, and it is possible that different bacteria could act as antagonists to a range of bacteria. In other circumstances bacteria could produce bacteriocins, microcins or enzymes, which under the right conditions could influence bacterial

attachment. Also in biofilm development in the natural environment, bacteria will attach on top of one another. Komiyama (1984) has demonstrated this, and its importance must be considered (Chapter 6).

In natural aquatic environments, many different organisms will be present. These organisms may influence bacterial biofilm development in various ways. In some ecosytems, phytoplankton are the major organic substrate for heterotrophic bacteria. Research has indicated that the physiological state of the phytoplankton can influence the attachment of the heterotrophic bacteria. The attachment of these bacteria increased at the same time as the phytoplankton growth started, but attachment decreased as the phytoplankton grew, and only when growth had stopped did attachment of the bacteria again increase. The attachment pattern demonstrated was due to a close coupling between the physiological state of the phytoplankton and their associated bacteria (Albright, 1986). Phytoplankton in natural aquatic environments have also been shown to produce antibiotic-like substances which could inhibit the growth and attachment of bacteria. If these substances were formed during the growth phase of the phytoplankton, this would account for the decrease in bacterial attachment observed (Cooper, 1983). Phytoplankton have also been shown to have cell-attached bacterial growth inhibitors which could influence bacteria and their attachment in natural aquatic environments (Cooper, 1983). This is a good example how one organism can inhibit or promote the attachment of a bacterium. It also demonstrates some of the different methods used to inhibit bacterial attachment in the natural aquatic environments.

Toxins produced in the natural environment have been shown to influence bacterial populations (Liang, 1982). Therefore, such

substances produced by different microorganisms in aquatic environments, could influence bacterial attachment. Other substances could be present in the natural aquatic environment which could influence attachment. Macromolecules which are present prior to bacterial attachment and involved in forming the conditioning layer (Chapter 1), can influence bacterial attachment. It is also possible that these macromolecules could influence bacterial attachment once the bacteria have attached to the surface. The overall importance of these macromolecules in attachment is questionable. Macromolecules have been shown not to influence bacterial attachment (Gordon, 1987), and in other instances bacterial attachment is decreased if macromolecules are present. It is likely that the effects of macromolecules on bacterial attachment are dependent on the bacteria under investigation. Abrahams (1983), showed that human buccal epithelial cells, rich in the macromolecule fibronectin, bound only Gram-negative bacteria and not Gram-positive bacteria. When fibronectin was not present Gram-positive bacteria were seen to attach, hence, this macromolecule influenced the attachment of bacteria to the cells.

Inorganic particles found in natural aquatic environments could not have entered the model system due to the membrane filters used. Gordon (1983) demonstrated that bacterial activity was not enhanced by particulate material. Bacteria, however, could attach to these particles if they were present in the model system, and this could result in decreased numbers of bacteria attaching to the glass surfaces in the model system.

Other members of the natural aquatic community will also be important in bacterial biofilm development. A good example of this is

the importance of predators, such as microflagellates, on bacterial populations in aquatic environments (Alexander, 1981). Heterotrophic microflagellates are often seen associated with particulate matter, (Caron, 1986; Davoll, 1983) and in some instances a high affinity for surfaces is exhibited by these protozoa, which is surprising as many species of microflagellates graze suspended bacteria (Davis,1984). Attached microflagellates have been shown to graze suspended bacteria, and attachment to surfaces is believed to increase the efficiency of the flagella movements used to create feeding currents (Fenchel, 1982). Caron (1987) investigated the grazing habits of four heterotrophic microflagellates on an attached and unattached Pseudomonas and showed that two of the microflagellate species grazed on the unattached bacteria but had no ability to graze on attached bacteria, whereas the other two microflagellates grazed on the attached Pseudomonas to a greater extent than the unattached Pseudomonas. In these studies the growth of the latter two species of microflagellates was seen to be related to the density of attached bacteria. Therefore, the presence of the microflagellates could be related to the distribution of suspended particles and their associated bacteria. In coastal marine environments it has been suggested that microflagellates utilise up to 60% of the bacterial biomass and, therefore, are very significant in these aquatic environments. The importance of these studies to freshwater aquatic habitats is questionable, however, the importance of grazing on bacterial biofilms in freshwater cannot be overlooked.

Apart from microflagellate grazing, the biomass of bacteria could also be influenced by other factors such as lysis. This lysis could be spontaneous or induced by bacterial predators or by bacteriophage

(Casida, 1980). In the experiments described here the importance of grazing or of of predation is questionable as the membranes used in the model system would prevent this. Autolysis or bacteriophage-induced lysis, however, could influence biofilm development in our model system. The bacteriophage population in rivers has been shown to vary with factors which also influence bacterial populations and bacterial attachment. These factors include temperature and particulate matter. It is possible that phage could influence biofilm development if their hosts were part of the bacterial biofilm concerned (Nuttall, 1987). As with much of this discussion however much more work is required to understand bacterial attachment in natural aquatic environments and the factors which influence that attachment.

CHAPTER EIGHT

AN INVESTIGATION INTO THE INHIBITION OF BACTERIAL ATTACHMENT BY ACINETOBACTER, AND THE EFFECTS OF DIFFERENT MOLECULAR WEIGHT LAKE WATER FRACTIONS ON THE ATTACHMENT OF BACTERIA.

8.1 AIM

To investigate bacterial attachment in the presence of Acinetobacter, the species which influenced the attachment of another species in a mixed bacterial suspension (Chapter 7). This study was designed to try and establish the mechanism of inhibition of attachment by Acinetobacter. The attachment of bacteria in the model system (Chapter 7) was harder to interpret than the bacterial attachment results obtained in batch culture. To aid the understanding of the results from the model system, the influence of different molecular weight fractions of Tocil Lake water on bacterial attachment was studied.

8.2 INTRODUCTION

In Chapter 7 Acinetobacter was found to reduce the attachment of the other members of a mixed suspension in all the cases studied. Experiments were therefore designed to try and establish the method by which Acinetobacter inhibited attachment of other species. It is possible that Acinetobacter physically stopped the other species from attaching to the surface. As Acinetobacter itself did not attach to the surface under any conditions, this inhibition was not due to physical hinderence. It is possible that Acinetobacter itself physically reacted with the other bacterial cells prior to attachment and altered their attachment ability. Acinetobacter could alternatively produce a chemical

which could influence the attachment of the other species. This chemical could be produced for this specific purpose, or, more realistically, produced by Acinetobacter for another purpose. In the latter case this chemical could even be a waste product, but to be effective the chemical would have to be secreted into the liquid medium, and not remain bound to the bacterial surface.

In the laboratory, the composition of the growth medium was shown to influence, bacterial attachment (Chapters 4 and 6). In the natural environment the composition of the attachment medium was hard to establish. For instance the chemical composition of dissolved organic carbon (DOC) in natural waters is incompletely characterised and therefore, its effect on bacterial attachment is hard to assess.

Tranvik (1990) and Meyer (1987) demonstrated that particular fractions of the DOC could influence the growth of bacteria, therefore, it is possible that different molecular weight fractions obtained from Tocil Lake water could influence bacterial attachment. Membranes with different pore sizes were used to remove specific fractions from Tocil lake water, and these different lake water fractions investigated for their influence on bacterial attachment.

8.3 MATERIALS AND METHODS

8.3.1 Bacteria

Stock cultures of Aeromonas, Chromobacterium. Staphylococcus and Acinetobacter were prepared in glucose minimal media as in Section 7.3.1.

8.3.2. (A) Inhibition of Bacterial Attachment by Acinetobacter

The mechanism by which Acinetobacter influenced the attachment of other species in the model system was investigated using batch culture

attachment experiments. Acinetobacter as in the model system did not attach during batch culture attachment experiments (Chapter 3). The attachment of other species in the presence of Acinetobacter could therefore be investigated in the laboratory where growth conditions could be controlled.

The mechanism by which Acinetobacter influenced bacterial attachment could be due to a chemical produced specifically for this purpose, or as part of another process e.g. a waste product. The latter would be produced even when another species was not in suspension with the Acinetobacter. but would be subject to nutritional control. If the substance was produced specifically to influence the attachment of other species, it would only be produced when another species was present. Alternatively, the Acinetobacter cell itself might have to be present to influence bacterial attachment. This could be due to a chemical on the surface of the Acinetobacter cell influencing attachment of other species, or due to Acinetobacter cells themselves interacting with the other species in the liquid phase, to inhibit attachment.

To investigate if Acinetobacter produced a substance which influenced the attachment of other bacterial species, samples of Aeromonas. Chromobacterium. Staphylococcus and Acinetobacter were grown in batch culture in glucose Medium 6 at 15°C until late stationary phase as in Chapter 4. Mixed suspensions of bacteria were prepared as in Chapter 4 with Acinetobacter in a mixed suspension with each of the other species. Attachment experiments were performed with each of these mixed bacterial suspensions for two hours (Section 3.3.4). Once attachment experiments were completed, the surfaces were removed and the bacterial cells were centrifuged at 10,000 av.g for 15 mins to seperate

them from the supernatant. This supernatant was kept for future attachment experiments. If Acinetobacter produced a substance which influenced bacterial attachment, it would be present in this supernatant.

Aeromonas, Chromobacterium and Staphylococcus were grown in batch culture in 90ml glucose Medium 6 at 15°C until late stationary phase. 10ml of the supernatant from the appropriate attachment experiment was added to each flask along with glass coverslips. Attachment experiments were performed for two hours, and the attachment of the bacterium compared to the attachment in the controls with no supernatant obtained from the above experiments.

The latter experiment investigated the supernatants during mixed suspension attachments with Acinetobacter, to determine if a chemical substance was produced by Acinetobacter to influence the attachment of other species when they were in a mixed suspension. To investigate if Acinetobacter cells had to be present to account for attachment inhibition, Acinetobacter cells were again grown in batch in glucose Medium 6 at 15°C to late stationary phase. These Acinetobacter cells were separated from the supernatant by centrifugation at 10,000 av.g for 15 mins. Acinetobacter cells were killed to prevent them producing chemicals to inhibit bacterial attachment by washing in 0.5% formaldehyde. These Acinetobacter cells were used in subsequent attachment experiments after being washed three times in 1M phospate buffer. Viable counts were taken before and after formaldehyde treatment indicated that the formaldehyde treatment was 99.99% successful at killing Acinetobacter.

Aeromonas, Chromobacterium and Staphylococcus were grown in glucose Medium 6, at 15°C in batch culture, until late stationary phase. These bacteria were then separated from the supernatant by centrifugation at 10,000 av.g for 15 mins. Equal numbers of Aeromonas, Chromobacterium and Staphylococcus were resuspended in the appropriate supernatant with the same number of killed Acinetobacter cells. Attachment experiments were then performed as in Section 3.3.4 after the addition of glass coverslips. After the attachment experiments were completed the surfaces were investigated as in Section 4.3.3.

8.3.2.(B) Effect of Lake Water Molecular Weight Fractions on Bacterial Attachment

The effect of different molecular weight fractions of Tocil Lake water on attachment of Aeromonas, Chromobacterium and Staphylococcus was investigated using three different water fractions. These water fractions were prepared using water samples taken from a constant water sampling point at Tocil Lake. For each set of water fractions, two litres of lake water were first filtered through 0.22 μm millipore filters to remove bacteria. Amicon ultrafiltration filters were prepared for use by soaking in ethanol for 24 hrs. These filters were washed extensively in sterile deionised water before use.

Lake water was filtered through an Amicon PM30, PM10 or YM2 membrane with nitrogen pressure (10 psi) until 50 ml of water remained. This was done in a cold room to retard bacterial growth. Three concentrated water fractions were produced, (a) > 30,000 molecular weight material, (b) 10,000-30,000 molecular weight material and (c) 1,000-10,000 molecular weight material (FIGURE 8.1).

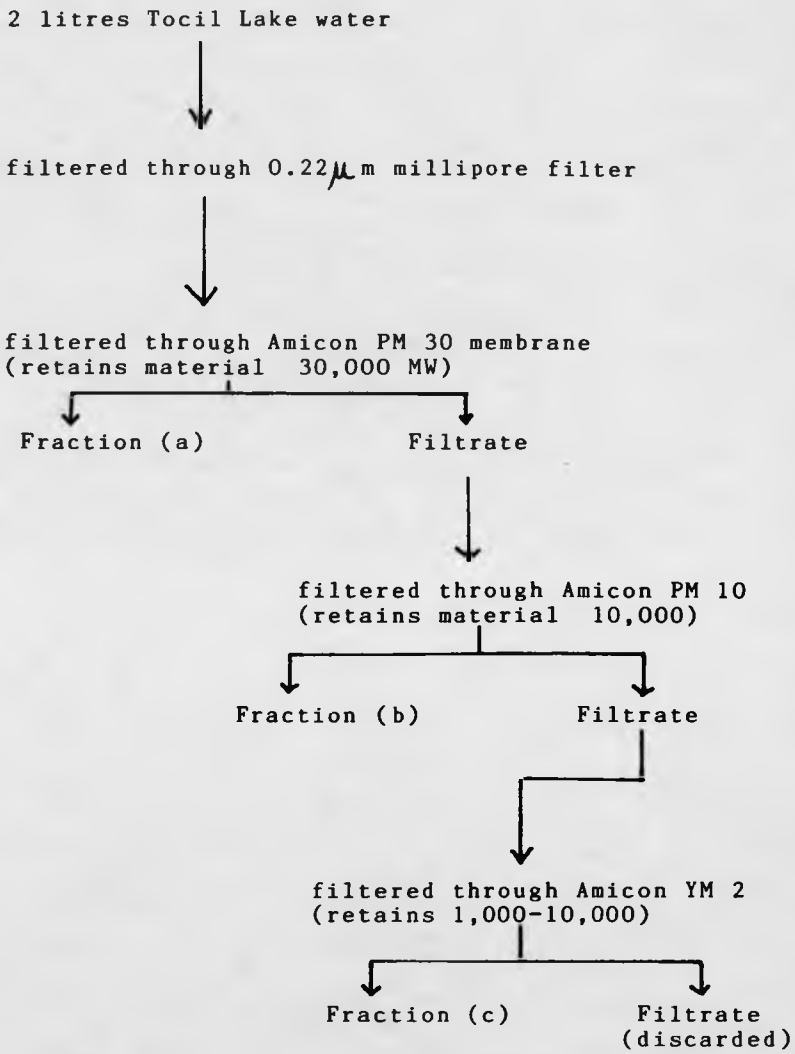


FIGURE 8.1
The filtration process employed to obtain water fractions (a), (b) and (c).

Aeromonas, Chromobacterium and Staphylococcus were grown in batch culture in 90 ml of glucose Medium 6 plus 10ml of each water fraction at 15°C until late stationary phase. Attachment experiments were performed as in Section 3.3.4. These bacteria were also grown in batch culture in 90ml of glucose Medium 6 at 15°C till late stationary phase without the presence of water fractions. Prior to the attachment experiments 10 ml of each water fraction was added to each of the 90 ml of glucose Medium 6 with the different bacteria to give a total volume of 100 ml. Controls were set up with each bacterium grown in batch culture in 100 ml of glucose Medium 6 at 15°C to late stationary phase. Attachment experiments were then performed as in Section 3.3.4. After attachment experiments were completed the surfaces were removed and investigated as in Section 4.3.3.

8.4 RESULTS

8.4.1.(A) Bacterial Attachment Inhibition by Acinetobacter

FIGURE 8.2 shows the results obtained from the investigation into the effects of Acinetobacter on bacterial attachment. When the attachment results for pure culture Aeromonas, Chromobacterium and Staphylococcus (FIGURE 8.2 (I)) were compared with the results obtained when Aeromonas, Chromobacterium and Staphylococcus were attached in a mixed suspension with Acinetobacter (FIGURE 8.2 (II)) the attachment in the presence of Acinetobacter was always significantly ($P>0.05$) lower than the attachment results for the pure cultures. In the case of Aeromonas and Chromobacterium, the decrease in attachment was greater than 50%. With Staphylococcus the decrease in attachment observed was approximately 40%. These results demonstrate that Acinetobacter could influence the attachment of another species, when present in a mixed

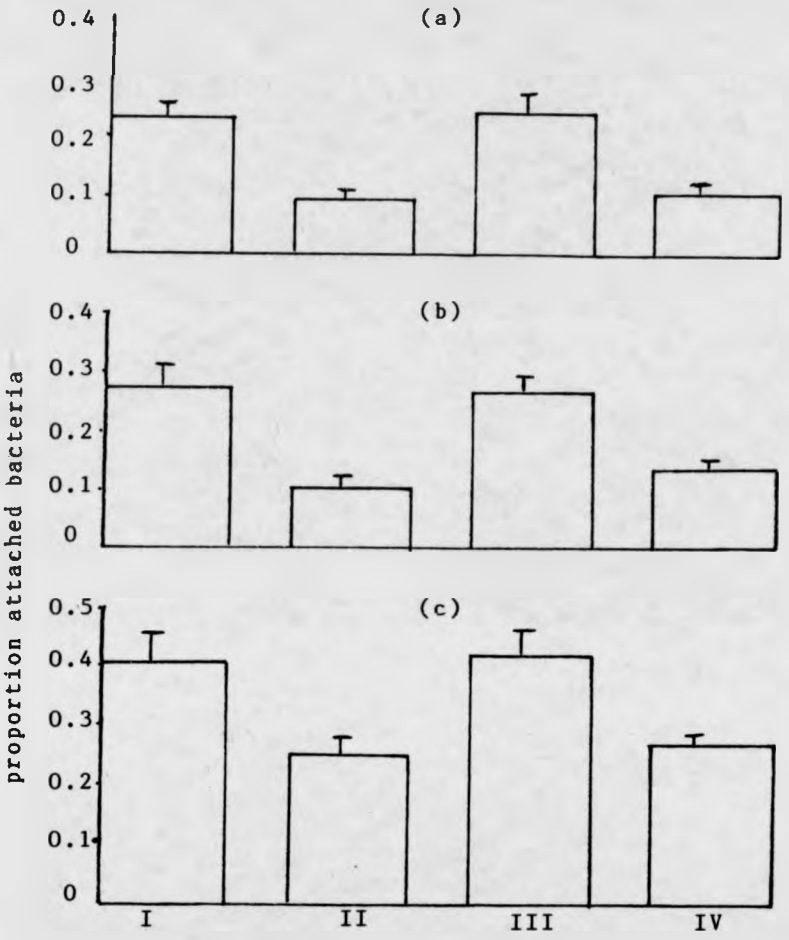


FIGURE 8.2
The attachment of (a) Aeromonas, (b) Chromobacterium and (c) Staphylococcus in;
(I) glucose medium 6,
(II) glucose medium 6 in a mixed suspension with Acinetobacter,
(III) glucose medium 6 with mixed suspension supernatent,
(IV) glucose medium 6 with killed Acinetobacter cells.

N.B.- The medium indicated is the growth medium and the attachment medium.
All cultures were grown at 15°C. The temperature at which attachment was carried out was 15°C.

suspension in batch, as it did in the model system experiments (Chapter 7).

The results obtained for the attachment of Aeromonas, Chromobacterium and Staphylococcus in the presence of the appropriate Acinetobacter supernatant results are shown in FIGURE 8.2 (III). It is clear that in each case this supernatant had little effect on the attachment of these species. The attachment results obtained were always comparable to the pure culture controls.

In contrast when Aeromonas, Chromobacterium and Staphylococcus were attached in the presence of killed Acinetobacter cells, changes in bacterial attachment were observed. Compared to the pure culture controls, the attachment of Aeromonas, Chromobacterium and Staphylococcus was significantly ($P>0.05$) lower in the presence of the killed Acinetobacter cells (FIGURE 8.2 (IV)). In each case the results obtained (FIGURE 8.2 (IV)) for the attachment of these bacteria in the presence of dead Acinetobacter cells were similar to the results obtained when these bacteria were attached in a mixed suspension with viable Acinetobacter cells (FIGURE 8.2 (II)). These results suggest that it is the Acinetobacter cells themselves which influenced bacterial attachment in these studies. It is unlikely that Acinetobacter excreted a chemical into the attachment medium to influence the attachment of other species, as this chemical would have been present in the supernatant used. However, it may be possible that such a chemical could be present on the surface of the Acinetobacter cell itself, or some other form of attachment inhibition may be present. Further studies of the mechanism involved in attachment inhibition by Acinetobacter must be performed before this becomes clear.

8.4.2 Effects of Water Fractions on Bacterial Attachment

The results obtained for the effect of different Tocil Lake water fractions on the attachment of Aeromonas, Chromobacterium and Staphylococcus are shown in FIGURES 8.3 and 8.4. As growth conditions can influence bacterial attachment (Chapter 4), water fractions were first investigated after being introduced to the growth medium. These water fractions did not influence the growth of bacteria investigated. In each case the growth of a bacterium in growth medium in the presence of a water fraction was comparable to that observed when bacteria were grown in growth medium without a water fraction (Table 8.1). FIGURE 8.3 shows the attachment results obtained from these studies. As expected changes in the attachment of the species were observed when the different water fractions were present in the growth medium. Aeromonas and Chromobacterium showed little change in their attachment in the presence of fraction (c) compared to the control values. With fraction (b) small changes in attachment of these bacteria were observed compared to the control value. However, when fraction (a) was present in the growth medium both the attachment of the Aeromonas and Chromobacterium decreased significantly ($P>0.05$) compared to the control values.

Staphylococcus also showed little change in its attachment when fraction (b) and (c) was present in the growth medium compared to the control value. With fraction (a) however, the attachment of the Staphylococcus increased compared to the control value. These results suggest that the presence of these different water fractions in the growth medium could influence bacterial attachment.

These water fractions were also introduced just prior to the attachment experiments. This was to investigate if the changes in

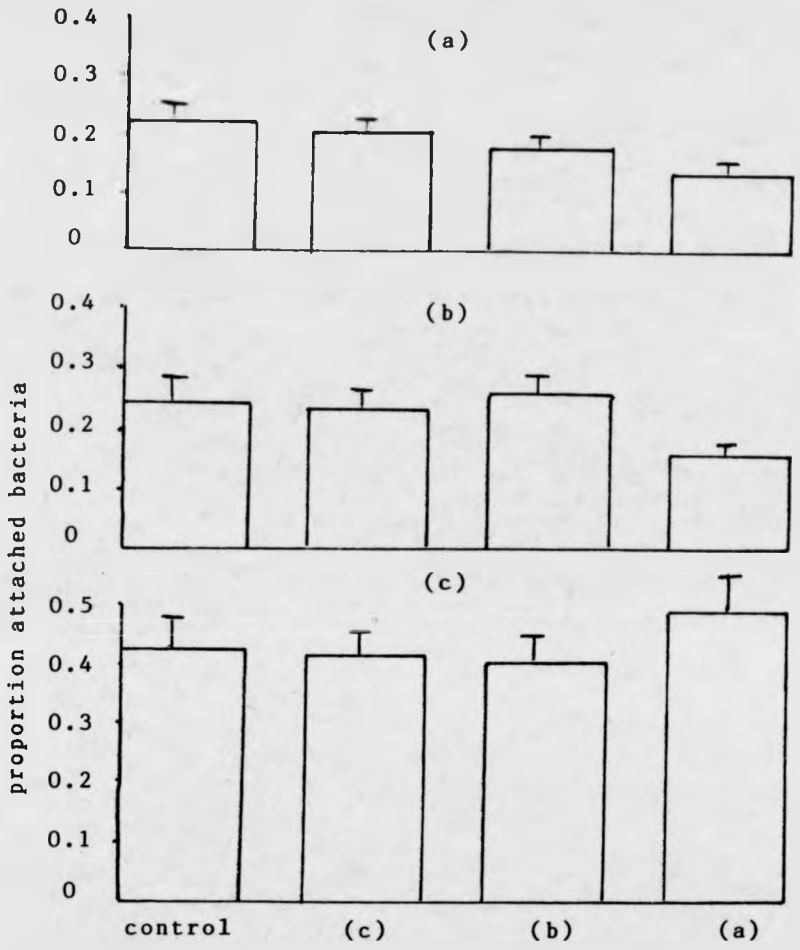


FIGURE 8.3
The attachment of (a) Aeromonas, (b) Chromobacterium and (c) Staphylococcus in;
(control)- glucose medium 6,
(c) - glucose medium 6 plus water fraction (c),
(b) - glucose medium 6 plus water fraction (b),
(a) - glucose medium 6 plus water fraction (a).

N.B.- The medium indicated is the growth medium and the attachment medium.
All cultures were grown at 15°C. The temperature at which attachment was carried out was 15°C.

attachment obtained occured due to changes in the bacteria during growth or if these fractions were influencing the attachment process. The results obtained when the water fractions were introduced prior to attachment are shown in FIGURE 8.4. Aeromonas and Chromobacterium attachment changed very little, if at all, in the presence of fractions (a), (b) or (c). These results are different to those obtained when the water fractions were introduced during growth. In the case of fraction (a) changes in bacterial attachment were not observed in these experiments although bacterial attachment was influenced when this fraction was introduced to the growth medium. These results suggest that this water fraction influences Aeromonas and Chromobacterium before attachment occurs and these changes probably to the cell surface occur during bacterial growth.

Staphylococcus showed similar results to those obtained with Aeromonas and Chromobacterium. With fraction (b) and (c), little change in the attachment of Staphylococcus was observed compared to the control value. Changes were observed, however, with fraction (a). The attachment of Staphylococcus significantly decreased compared to the control value. This was the opposite to the results obtained when fraction (a) was introduced into the growth medium. It was also different from the results obtained with Aeromonas and Chromobacterium with the water fractions introduced prior to attachment, where little change in attachment of these bacteria were observed. These results suggest that fraction (a) influences the attachment of Staphylococcus both during growth and prior to attachment.

These water fractions did not appear to influence the viability of the cells present in the attachment or growth medium. All bacterial

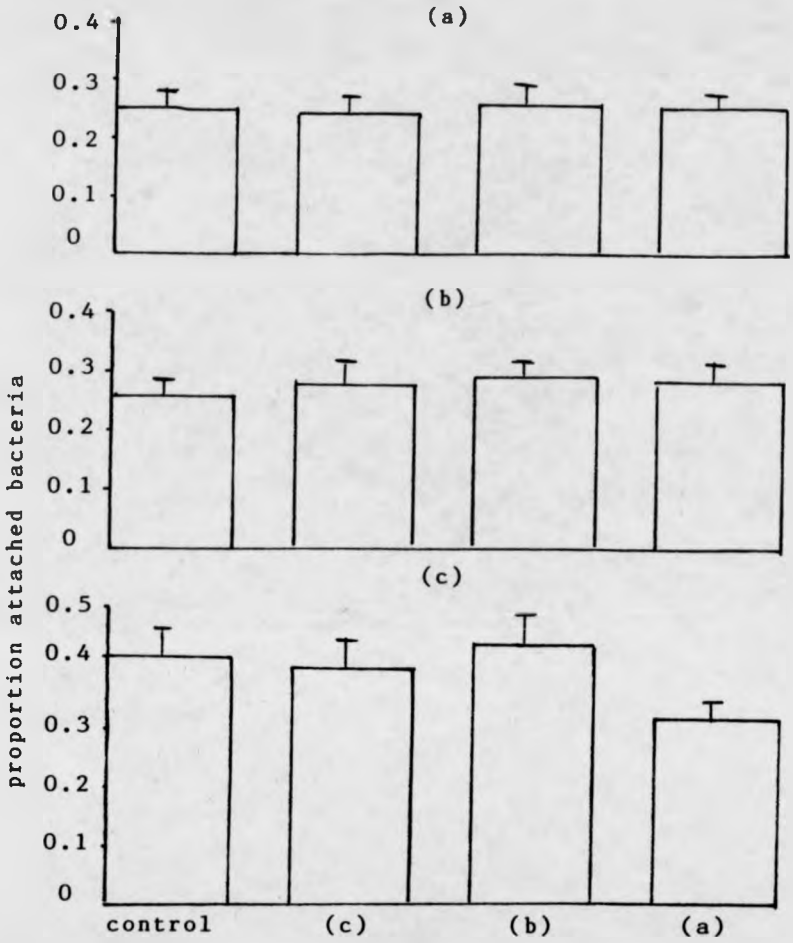


FIGURE 8.4
The attachment of (a) Aeromonas.(b) Chromobacterium and (c) Staphylococcus in;
(control)- glucose medium 6,
(c) - glucose medium 6 plus water fraction (c),
(b) - glucose medium 6 plus water fraction (b),
(a) - glucose medium 6 plus water fraction (a).

N.B.- The medium indicated is the growth medium only. All cultures were grown in glucose medium 6. Prior to attachment experiments the water fractions (a), (b) and (c) were added to the growth medium. The attachment experiments were then carried out at 15° C.

cells could be grown after these attachment experiments. The mechanism by which these water fractions influenced bacterial attachment is again unclear. As fraction (a) was seen to influence the attachment of the bacteria during growth and prior to attachment, it is possible that this water fraction influences bacterial attachment in a number of different ways. It is clear these water fractions and their effects on bacterial attachment have to be studied in more detail.

8.5 DISCUSSION

The results obtained from the investigation into the inhibition of attachment of other species by Acinetobacter suggest that a chemical substance, produced by Acinetobacter, is not responsible for this inhibition. As Acinetobacter does not attach to the surfaces, the interaction which causes the inhibition is not due to competition between the species for attachment sites. Inhibition only occurs when Acinetobacter cells are present and possibly involves some interaction between the cells in the liquid environment that prevents the cells attaching.

As discussed in Chapter 6 bacteria can greatly influence other bacterial species when present in the same environment by the production of specific substances like bacteriocins and extracellular enzymes. It is not known whether Acinetobacter produces any of these substances. Aeromonas hydrophila, a natural inhabitant of freshwater, has been shown to produce an enzyme which can inactivate its own adhesin (Adams, 1983). Imam (1990) has also demonstrated that a five day old culture of Arthrobacter contains an enzyme which can inhibit the adhesin of this bacteria. If such chemicals are produced by Acinetobacter then they could influence the attachment of other bacterial species. These

extracellular substances would be released from the cells into the medium and unlikley to be held close to the bacterial surface, but as no inhibition was caused by the culture supernatants it seems unlikely that such extracellular material was involved. Rollinger (1987) has shown that less than 1% of the microbial population, isolated from activated carbon, produced substances which inhibited the growth of other bacteria. This indicates that few bacteria produce substances which can inhibit the growth and attachment of other bacterial species. In other habitats, this has also been noticed with only one bacterial species in 34 being able to influence the growth of Haemophilus (Riley, 1986).

Cooper (1983) has shown that phytoplankton have cell-attached growth inhibitors which influenced the growth and attachment of bacteria. If such cell-attached inhibitors were present on the Acinetobacter cells, these could influence the attachment of the other bacterial species in the attachment experiments. In Chapter 6, an example of bacteria acting as receptors for the attachment of other species was given (Slots, 1978). Therefore, it is possible that bacterial cells could inhibit the attachment of other bacterial species by having inhibitors on their surface, but equally some unknown method could be involved.

Substances which are associated with bacterial surfaces such as polymers (Corpe, 1973) and slimes (Costerton, 1978) have been shown to be involved in bacterial attachment. These substances have also been shown not to be involved in bacterial attachment (Uhilinger, 1983). The function of the polymers not involved in attachment is unknown, but they could be involved in influencing other bacterial species coming into contact with the bacterium producing these polymers. This could involve influencing the attachment of these bacteria in some unknown way. These

polymers could have been developed for a specific purpose such as attachment, however, they could in turn inhibit the attachment of other bacteria instead of promoting it. Mutations in the genes coding for polymers are known (Pringle, 1983; Filloux, 1987: Deflaun, 1990), therefore it is possible that mutations can occur which results in a change in the function of the polymers coded for.

The formation and chemical content of extracellular polymers produced by bacteria can be influenced by growth conditions (Yoshikawa, 1979) and by environmental factors such as temperature (Fyfe, 1987). These observations suggest that changing these parameters will lead to changes in the polymers and possibly alterations to their functions. In our experiments, the extent of the effects of Acinetobacter on the attachment of other bacteria was less profound as the temperature of the model system increased (Chapter 7). These results suggest that growth and environmental conditions will influence the inhibition of bacterial attachment by other bacterial species as well as influencing the attachment of different bacterial species.

The results obtained from investigating the effects of different fractions of Tocil Lake water on the attachment of bacteria suggest that fraction (a) (containing >30,000 MW components) could influence bacterial attachment. When this water fraction was introduced, prior to growth, changes in the attachment of the bacterial species were observed (FIGURE 8.2). The variations observed in bacterial attachment depended on the bacterial species being investigated. With Aeromonas and Staphylococcus fraction (a) decreased attachment. The method by which this water fraction influenced bacterial attachment, without influencing bacterial growth, remains unclear. If part of this fraction was used as

a nutrient source then it could be linked to factors such as changes in the surface characteristics of the bacterial cells discussed in Chapter 5. Meyer (1987) used water fractions to investigate bacterial growth on DOC. Bacterial growth was greatest with the low molecular weight (MW) fraction (< 1,000 MW), least in the intermediate MW fraction (1,000-10,000 MW) and the high MW fraction (> 30,000 MW) supported more growth than the intermediate MW fraction. The latter was thought to be due to lower MW compounds complexed with a high MW refactory core. As bacterial growth was not influenced by these water fractions in the experiments reported here, possibly due to the bacteria using the nutrients present in the growth medium, it is possible that these fractions influenced bacterial attachment by a mechanism not involved with growth.

When the water fractions were introduced just prior to the attachment experiments (FIGURE 8.3), the results obtained did not compare to those obtained when the fractions were introduced, prior to growth (FIGURE 8.2). Only the attachment of Staphylococcus was influenced when the water fractions were introduced prior to attachment. It is unlikely that the physiology of Staphylococcus would have been influenced to any extent in the time period between addition of the fraction and commencement of the attachment experiment. This suggests that these water fractions could influence bacterial attachment in more than one way or that more than one substance in these fractions could influence bacterial attachment.

Theodore (1986) demonstrated that the presence of toxic compounds in lake water could cause a decrease in the density of bacteria introduced into this lake water. The decline in bacterial density was reduced with the presence of glucose in the medium. As glucose was present in the

medium used here, the affects of the inhibitors with regard to growth could have been reduced or been non-existent. These inhibitors could, however, still influence bacterial attachment if present. In these studies the bactericidal agents were thought to be low molecular weight compounds. This would not fit in with our experimental results where a high MW compound was involved in influencing bacterial attachment, although it is possible that low molecular weight compounds were complexed with the high molecular weight material. The method of producing the fractions by retaining the high molecular weight fraction on the filter, and then producing the other fractions from the supernatant, could have removed all low molecular weight material by complexing with the colloidal high molecular weight material. No check was made on the concentration of high and low molecular weight materials present in the relevant fractions. However, high MW compounds have been shown to influence bacterial growth in seawater (Saz, 1963; Massey, 1970). These high MW compounds could also be influenced by environmental conditions such as pH (Theodore, 1986) and also by seasonal variation (Moebus, 1972). The affects of such compounds on bacterial attachment would, therefore, be influenced by these factors. These compounds would also be expected to affect different bacterial species in different ways.

These results suggest that a great deal of research is required into the mechanism by which Acinetobacter influences the attachment of other bacterial species. Research is also required into different MW water fractions to try and establish the compounds in these fractions which influence bacterial attachment.

TABLE 8.1 Viable counts obtained when a bacterium was grown in glucose Medium 6 and in glucose Medium 6 with lake water fraction (a), (b) or (c).

BACTERIUM	WATER FRACTION			
	None	(a)	(b)	(c)
Aeromonas	3.2×10^8	2.9×10^8	3.5×10^8	2.5×10^8
Chromobacterium	1.6×10^8	1.2×10^8	2.0×10^8	1.3×10^8
Staphylococcus	5.9×10^7	5.1×10^7	5.0×10^7	4.9×10^7

CHAPTER NINE

CONCLUSIONS

The bacteria isolated from Tocil Lake (Chapter 2) can be classified as copiotrophic bacteria. These copiotrophic bacteria were used for the attachment experiment studies in this thesis as these bacteria have been reported to be the original colonisers of surfaces in aquatic environments. The fact that these bacteria could also be maintained easily in the laboratory was an important consideration in their selection.

The experiments performed and the results obtained in Chapter 3 emphasise the importance of controlling experimental procedures such as subculturing during attachment experiments. The bacterial concentration used in the attachment experiments was also important, as different species were shown to have different optimum concentrations for attachment, above which no increase in bacterial attachment with increasing numbers of bacteria occurred. If this optimum bacterial concentration was used in attachment experiments, increases in bacterial attachment due to the additional experimental parameters being studied would not be apparent. Therefore Chapter 3 outlined the experimental parameters which had to be controlled to ensure that the results obtained reflected the attachment experiments performed.

During the attachment experiments, the attached bacteria were sometimes hard to count microscopically on glass coverslips. Therefore attached bacteria were estimated in Chapters 4-8 using the detachment procedure outlined in Chapter 3. The attachment experiments performed in Chapters 4 and 5 emphasised how nutrient conditions such as carbon source and environmental conditions such as pH and temperature could

influence the attachment of pure cultures of bacteria to glass coverslips. These attachment experiments were also used in Chapter 6 with mixed bacterial suspensions. Therefore the results from Chapters 4 and 5 were used as controls for the mixed suspension attachment experiments in Chapter 6.

The results from Chapter 6 demonstrate that one bacterial species can influence the attachment of another bacterial species when they are attached to a solid surface in a mixed suspension. These results were dependent upon the bacterial species being investigated and the growth conditions used, as would be expected from the results obtained in Chapters 4 and 5.

In the laboratory, mixed bacterial suspensions containing two bacterial species were investigated. To investigate how an initial biofilm would develop in a natural aquatic environment, a model system was developed to study the attachment of mixed bacterial suspensions containing up to six bacterial species. The attachment results obtained in Chapter 7 show that different bacterial species can influence the attachment of other bacterial species when they are present in the model system. The results obtained in the model system were influenced by growth conditions as was demonstrated by the model system temperature experiments. The biofilms obtained in the model system when 3-6 bacterial species were present in the mixed suspensions show that a dominant bacterium was present in each biofilm. These results suggest that the dominant bacterium in each case is best suited for attachment to solid surfaces under the growth conditions provided.

The results in Chapter 7 show that Acinetobacter could influence the attachment of other bacterial species to a solid surface without

attaching to the solid surface itself. The mechanism used by Acinetobacter to influence bacterial attachment was investigated in the laboratory (Chapter 8). These studies suggest that the mechanism used by Acinetobacter to influence bacterial attachment was cell orientated, either bound to the cell surface or originating from inside the Acinetobacter cells. The inhibition of bacterial attachment by Acinetobacter was also influenced by temperature. However, the mechanism used by Acinetobacter to influence bacterial attachment must be studied in greater detail to understand more fully what is happening.

The nutrients entering the model system in Chapter 7 were thought to enter the system by diffusion. In Chapter 8 the water from Tocil Lake was investigated by extracting different molecular-weight water fractions to see how these different fractions influenced bacterial attachment. The results obtained from Chapter 4 on the effects of nutrient conditions on bacterial attachment suggested that these different fractions should influence bacterial attachment in different ways. The results obtained however, show that only the >30,000 MW fraction influenced bacterial attachment significantly. This >30,000 MW fraction was seen to increase or decrease the attachment of Staphylococcus, suggesting different mechanisms of action. However, this large MW water fraction could contain other smaller MW fractions mixed within its complex and will have to be investigated in greater detail to assess how it influences bacterial attachment to solid surfaces.

BIBLIOGRAPHY

Abraham, S., Beachey, E.H. and Simpson, W.A. 1983. Adherence of Streptococcus pyogenes, Escherichia coli and Pseudomonas aeruginosa to fibronectin-coated and uncoated epithelial cells. Infection and Immunity. 41: 1261-1268.

Absolom, D.R., Lamberti, F.V., Policova, Z., Zingg, W., van Oss, C.J. and Neumann, A.W. 1983. Surface thermodynamics of bacterial adhesion. Applied and Environmental Microbiology. 46: 90-97.

Adams, D., Atkinson, H.M. and Woods, W.H. 1983. The differing effect of proteases on the adhesions of Aeromonas hydrophila. FEMS Microbiology Letters. 20: 197-200.

Akagi, Y., Taga, N. and Simidu, V. 1977. Isolation and distribution of oligotrophic marine bacteria. Can. J. Microbiol. 23: 981-987.

Albright, L.J., McCabe, S.K. and May, B.E. 1986. Attached and free-floating bacterioplankton in Howe Sound, British Columbia, a coastal marine fjord-embayment. Applied and Environmental Microbiology. 51: 614-621.

Alexander, M. 1981. Why microbial predators and parasites do not eliminate their prey and hosts. Ann. Rev. Microbiol. 35: 113-133.

Asensio, C., Perez-Diaz, J.C., Martinez, M.C. and Baquero, F. 1976. Biochem. Biophysics. Rus. Communication. 69: 7-14.

Atkinson, B. 1974. Biochemical Reactors. Pion Limited, London.

Baier, R.E. 1980. Substrate influences on adhesion of microorganisms and their resultant new surface properties. In adsorption of microorganisms to surfaces. ed. G. Bilton and K.C. Marshall. 59-104. New. York. John Wiley and Sons.

Baier, R.E. 1981. Early events of micro-fouling of heat transfer equipment. 293-304. In E.S.C. Somerscales and J.G. Knudsen. ed. Fouling of heat transfer equipment. Hemi-sphere publishing Co. Washington D.C.

Baker, J.H. 1984. Factors affecting the bacterial colonization of various surfaces in a river. Can. J. Microbiol. 30: 511-515.

Ball, H. 1986. Personnel Communication. Warwick University.

Bell, C.R. and Albright, L.J. 1982. Attached and free-floating bacteria in a diverse selection of water bodies. Applied and Environmental Microbiology. 43: 1227-1237.

Ben-Nain, A. 1980. Hydrophobic Interactions. New York. Plenum Publishing Corporation.

Berger, L.R. and Berger, J.A. 1986. Countermeasures to microfouling in simulated ocean thermal energy conversion heat exchanges with surface and deep ocean waters in Hawaii. Applied and Environmental Microbiolgy. 51: 1186-1198.

Beverley, H., Kjelleberg, S. and Marshall, K.C. 1983. Responses of marine bacteria under starving conditions at a solid-water interphase. Applied and Environmental Microbiology. 45: 43-47.

Biddanola, B.A. 1985. Microbial synthesis of macroparticulate matter. Mar. Ecol. Prog. Ser. 20: 241-251.

Blazevic, D.T. and Grace, M.E. 1975. Principles of biochemical tests in diagnostic microbiology techniques. Pure and Applied Microbiology, London, John Wiley and Son.

Bratbak, G. 1985. Bacterial biovolume and biomass estimations. Applied and Environmental Microbiology 49: 1488-1493.

Bright, J.T. and Fletcher, M. 1983. Amino-acid assimilation and electron transport system activity in attached and free-living marine bacteria. Applied and Environmental Microbiology. 45: 815-825.

Brown, C.M., Ellwood, D.C. and Hunter, J.M. 1977. Growth of bacteria at surfaces: influence of nutrient limitation. FEMS. Microbiol. Letters. 1: 163-166.

Bryers, J.D. and Characklis, W.G. 1981. Kinetics of initial biofilm formation within a turbulent flow system in fouling of heat transfer equipment. ed. E.F.C. Somerscales and J.G. Knudsen. 313-333. Hemisphere Publishing Company. Washington.

Burkholder, J.M., Wetzel, R.G. and Klomparens, K.L. 1990. Direct comparison of phosphate uptake by adnate and loosely attached microalgae within an intact biofilm matrix. Applied and Environmental Microbiology. 56: 2882-2890.

Bush, P.L. and Stumm, W. 1968. Chemical interactions in the aggregation of bacteria: bioflocculation waste treatment. En. Sci. Tech. 2: 49-53

Caldwell, D.E. and Lawrence, J.R. 1986. Growth kineti of Pseudomonas fluorescens microcolonies within the hydrodynamic boundary layers of surface microenvironments. Microbiol. Ecol. 12: 299-312.

Cammen, L.M. and Walker, J.A. 1982. Distribution and activity of attached and free-living suspended bacteria in the Bay of Fudy. Can. J. Fisln. Ag. Sci. 39: 1655-1663.

Caron, D.A., Davis, P.G., Madin, L.P. and Sieburth, J. McN. 1986. Enrichment of microbial populations in macroaggregates (marine snow) from surface waters of North Atlantic. J. Mar. Res. 44: 543:564.

Caron, D.A. 1987. Grazing of attached bacteria by heterotrophic microflagellates. Microb. Ecol. 13: 203-218.

Carrie, A.R. and Goulder, R. 1985. A note on the attachment rate of suspended bacteria to submerged aquatic plants in a calcareous stream. J. of Applied Bacteriol. 59: 389-392.

Casida, L.E. 1980. Bacterial predators of Micrococcus luteus in soil. Applied and Environmental Microbiology. 39: 1035-1041.

Cerini, W.F., Battles, W.R. and Jones, P.H. 1946. Some factors influencing the plugging characteristics of an oil-well injection water. 52-63. Petroleum Technology Publication 2028. American Institute of Mining Engineers.

Characklis, W.G. and Cooksey, K.E. 1983. Biofilms and microbial fouling. Advances in Applied Microbiology. 29: 93-138.

Chung, K.T., Dickson, J.S. and Crouse, J.D. 1989. Effects of nisin on the growth of bacteria attachment to meat. Applied and Environmental Microbiology. 55: 1329-1333.

Clarke, K.R. and Joint, I.R. 1986. Methodology for estimating the numbers of free-living and attached bacteria in estuarine water. Applied and Environmental Microbiology. 51: 1110-1120.

Clenentz, D.M., Palterson, D.E., Aseltine, R.J. and Young, R.E. 1982. Stimulation of water injection wells in the L.A. Basin by using Sodium hypochlorite and mineral acids. J. Pet. Technol. 34: 287-296.

Cooper, S., Battat, A., Marsot, P. and Sylvestre, M. 1983. Production of antimicrobial activities by two Bacillaiophyceae. Can. J. Microbiol. 29: 338-341.

Corpe, W.A. 1973. Microfouling the role of primary film forming bacteria. Proceedings of the third International Congression marine-corrosion and fouling. ed. R.F. Acher, B.F. Brown, J.R. Depolma and W.P. Iverson. 598-609. Illinois, Northwestern University Press. Evanston.

Corpe, W.A., Matsuuchi, L. and Armbruster, B. 1975. Secretion of adhesive polymers and attachment of marine bacteria to surfaces. Proceedings of third International Symposium. ed. J.M. Sharpley and A.M. Kaplon. 433-443. London. Applied Science Publishers.

Costerton, J.W., Geesey, G.G. and Cheng, K.J. 1978. How bacteria stick. Scientific American. 238: 86-95.

Crisp, D.J. and Ryland, S. 1960. Influence of filming and surface texture on the settlement of marine organisms. Nature. 185: 119-121.

Cundell, A.M., Sleeter, T.D. and MItchell, R. 1977. Microbial populations associated with the surface of the brown algae Ascophyliumnodosum. Microbiol. Ecol. 4: 81-91.

Dahle, A.B. and Laake, M. 1982. Diversity dynamics of marine bacteria studied by immunofluorescent staining on membrane filters. Applied and Environmental Microbiology. 43: 169-176.

Daniels, S.L. 1980. Mechanisms involved in sorption of microorganisms to solid surfaces. In adsorption of microorganisms to surfaces. ed. G. Briton and K.C. Marshall. 8-58. New York. Wiley and Sons.

Danielsson, A., Norkrans, B. and Bjornsson, A. 1977. Bacterial adhesion and the effect of certain enzymes on adhered cells of a marine Pseudomonas species. Botanica Marina. 20: 13-17.

Davis, P.G. and Sieburth, J. McN. 1984. Predation of actively growing bacterial populations by estuarine and oceonic microflagellates. Mar. Ecol. Prog. Ser. 19: 237-246.

Davoll. P.G. 1983. Life history and community structure of marine snow aggregates from Monterey Bay. (Abstr). Ecos. 64: 1020.

Dawson, M.P., Humphrey, B. and Marshall, K.C. 1981. Adhesion a tatic in the survival stratergy of a marine vibrio during starvation. Curr. Microbiol. 6: 195-198.

DeFlaun, M.F., Tanza, A.S., McAteer, A.L., Marshall, B. and Levy, S.B. 1990. Development of an adhesion assay and characterization of an adhesion-deficient mutant of Pseudomonas fluorescens. Applied and Environmental Microbiology. 56: 112-119.

Dempsey, M.J. 1981. Marine bacterial fouling, a scanning electron microscope study. Marine. Biology. 61: 305-315.

Doyle, R.J., Nesbitt, W.E. and Grant-Taylor, K. 1982. The mechanisms of adherence of Streptococcus sanguis to hydroxylapatite. FEMS. Microbiol. Letters. 15: 1-5.

Ducklow, H.W., Kirchman, D.L. and Rowe, G.T. 1982. Production and vertical flux of attached bacteria in the Hudson River. Applied and Environmental Microbiology. 43: 769-776.

Duff, S.J.B., Copper, D.J. and Fuller, M. 1985. Effects of colloidal materials on cellulase production by Trichoderma reesei Rut-C30. Applied and Environmental Microbiology. 49: 934-938.

Duguid, J.P. and Wilkinson, J.F. 1953. The influence of culture conditions on polymer production by Aerobacter aerogenes. J. of Gen. Microbiol. 9: 174-189.

Duguid, J.P. and Gillies, R.R. 1957. Fimbriae and adhesive properties of dysentery bacilli. J. Pathol. Bacteriol. 74: 397-411.

Duguid, J.P., Clegg, S. and Wilson M.R. 1979. The fimbrial and non-fimbrial haemagglutinins of E. coli. J. Med. Microbiol. 12: 213-227.

Eighmy, T.T. and Bishop, P.L. 1985. The effect of turbulence on the binding-protein-mediated aspartate transport system in thin waste water biofilms. Applied and Environmental Microbiology. 50: 120-129.

Ekback, G., Morner, S., Lund, B. and Normark, S. 1986. Correlation of genes in the pap gene cluster to expression of globoside specific adhesion by uropathogenic E. coli. FEMS. Microbiol. Letters. 34: 355-360.

Ellwood, D.C. and Tempest, D.W. 1972. The effects of the environment on bacterial wall content and compostion. Advances in Microbiol Physiology. 7: 83-117.

Ellwood, D.C., Keevil, E.W., Marsh, P.D., Brown, C.M. and Wardell, J.N. 1982. Surface associated growth. Philosophical transactions of the Royal Society, London. 297: 517-532.

Ensign, J.C. and Wolfe, R.S. 1974. Nutritional control of morphogensis in Arthrobacter. J. of Bacteriol. 87: 924-932.

Esener, A.A., Roels, J.A. and Kossen, N.W.F. 1982. Dependance of the elemental composition of Klebsiella pneumoniae on the steady-state specific growth rate. Biotech. and Bioeng. 24: 1445-1449.

Fader, R.C., Houston, C.W. and Davis, C.P. 1984. Inhibition of bacteria adherence to rat bladder epithelial cells by human immune serum globulin, Curr Microbiol. 10: 29-34.

Feldner, J., Bredt, W. and Kahane, I. 1983. Influence of cell shape and surface charge on the attachment of Mycoplasma pneumoniae to glass surfaces. J. of Bacteriol. 153: 1-5.

Fenchel, T. 1982a. Ecology of heterotrophic microflagellates: 1: Some important forms and their functional morphology. Mar. Ecol. Prog. Ser. 8: 211-223.

Fenchel, T. 1982b. Ecology of heterotrophic microflagellates: 3: adaptations to heterogenous environments. Mar. Ecol. Prog. Ser. 9: 25-33.

Fenchel, T. 1982c. Ecology fo heterotrophic microflagellates: 4: quantative occurrence and importance as consumers of bacteria. Mar. Ecol. Prog. Ser. 9: 35-42.

Ferguson, R.L. and Rublee, P. 1976. Contribution of bacteria to the standing crop of coastal palnkton. Limnol Oceanogr. 21: 141-144.

Filloux, A., Murgier, M., Wretlind, B. and Lazdunski, A. 1987. Characterisation of two Pseudomonas aeruginosa mutants with defective secretion of extracellular proteins and comparison with other mutants. FEMS Microbiology Letters. 40: 159-163.

Finegold, S.M., Martin, W.J. and Scott, E.G. 1978. Bailey and Scotts diagnostic microbiology. 413-420. The C.V. Moslay Co. St. Louis.

Fletcher, M. and Floodgate, G.D. 1972. An electron-microscope demonstration of an acidic polymer involved in the adhesion of a marine bacterium to solid surfaces. J. Gen. Microbiol. 74: 325-334.

Fletcher, M. 1976a. The effects of proteins on bacteria attachment to polystyrene. J. of Gen. Microbiol. 94: 400-404.

Fletcher, M. 1976b. The effects of culture concentration and age, time and temperature on bacterial attachment to polystyrene. Can. J. of Microbiol. 23: 1-6.

Fletcher, M. and Loeb, G.I. 1979. Influence of substratum characteristics on the attachment of a marine Pseudomonad to solid surfaces. Applied and Environmental Micorbiology. 37: 67-72.

Fletcher, M. 1979. A microautoradiaographic study of the activity of attached and free-living bacteria. Arch. Microbiol. 122: 271-274.

Fletcher, M. 1980. Adherence of marine microorganisms to smooth surfaces. In Bacterial Adherence, ed. E.H. Beachey, 345-374. London: Chapman & Hall.

Fletcher, M and Marshall, K.C. 1982. Are solid surfaces of ecological significance to aquatic bacteria. Advances in microbiol ecology. 6: 199-235.

Fletcher, M. 1983. The effects of methanol, ethanol, propanol and butanol on bacterial attachment to surfaces. J. of Gen. Microbiol. 129: 633-641.

Fletcher, M. and Pringle, J.H. 1983. The effect of surface free energy and medium surface tension on bacterial attachment to solid surfaces. J. of Colloid and Interface Science. 104: 5-13.

Fletcher, M. 1984. Comparative physiology of attached and free-living bacteria. In: Microbial adhesion and aggregation. 223-232. ed. K.C. Marshall and D. Konferenzen. 1984. Springer-verlag. Berlin, New York.

Fletcher, M. and Pringle, J.H. 1985. Effect of surface free energy and medium surface tension on bacterial attachment to solid surfaces. J. Colloid and Interfaces. 104: 5-14.

Fletcher, M. 1986. Measurement of glucose utilisation by Pseudomonas fluorescens, that are free-living and that are attached to surfaces. Applied Environmental Microbiology. 52: 672-676.

Flint, K.P. 1985. A note on a selective agar medium for the enumeration of Flavobacterium species in water. J. of Applied Bacteriol. 59: 561-566.

Fontana, R., Canepari, P. and Satta, G. 1979. Alterations in peptidoglycan chemical composition associated with rod-to-sphere transition in a conditional mutant of Klebsiella pneumoniae. J. of Bacteriol. 139: 1028-1038.

Fraleigh, S.P. and Bungry, H.R. 1986. Modelling of nutrient gradients in a bacterial colony. J. of G. Microbiol. 132: 2057-2060.

Fry, J.C. and Zia, T. 1982. A method for estimating viability of aquatic bacteria by slide culture. Journal of App. Bacteriol. 53: 189-198.

Fyfe, L., Coleman, G. and Munroe, A.L.S. 1986. A comparison of the distribution of extracellular proteins by the protease-secreting organism Aeromonas salmonicida during aerobic and anaerobic growth. Annales de l'Institut Pasteur Microbiologie. 137A: 117-123.

Fyfe, L., Coleman, G. and Munroe, A.L.S. 1987. A comparative study of the formation of exrtracellular proteins by Aeromonas salmonicida at two different temperatures. J. of App. Bacteriol. 62: 367-370.

Gabriel Piette, J.P. and Idziak, E.S. 1991. Role of flagella in adhesion of Pseudomonas fluorescens to tendon slices. Applied and Environmental Microbiology. 57: 1635-1639.

Geesey, G.G. and Costerton, J.M. 1979. Microbiology of a northern river: bacterial distribution and relationship to suspended sediment and organic carbon. Can. J. Microbiol. 25:1058-1062.

Gerson, D.F. and Scheer, D. 1980. Cell surface energy, contact angles and phase partition III. Adhesion of bacterial cells to hydrophobic surfaces. Biochimica et Biophysica Acta. 602: 506-510.

Gingell, D. 1978. Experimental study of cell adhesion. In Ions in Macromolecular and Biological Systems, ed. D. H. Everett and B. Vincent. 237-241. Colsten Papers 29. Bristol. Scientechnica.

Gong, J. and Forsberg, C.W. 1989. Factors affecting adhesion Fibrobacter succinogenes subsp. succininogenes S85 and adherence-defective mutants to cellulose. Applied and Environmental Microbiology. 55: 3039-3044.

Gordon, A.S., Gerchakov, S.M. and Millero, F.J. 1983. Effects of inorganic particles on metablism by a periphytic marine bacterium. Applied and Environmental Microbiology. 45: 411-419.

Gordon, A.S. and Millero, F.J. 1985. Adsorption mediated decrease in the biodegradation rate of organic compounds. Microb. Ecol. 11: 289-298.

Gordon, A.S. 1987. Influence of alginate on attachment of Vibrio species to stainless steel surfaces in seawater. App. and Env. Microbiol. 53: 1175-1177.

Gorman, S.P., McCafferty, D.F., Woolfson, A.D. and Jones, D.S. 1987a. Reduced adherence of microorganisms to human muccasal epithelial cells following treatment with taurolin a novel antimicorbial agent. J. of Applied Bacteriol. 62: 315-320.

Gorman, S.P., McCafferty, D.F., Woolfson, A.D. and Jones, D.S. 1987b. Electron and light microscope observations of bacterial cell surface effects due to taevolioline treatment. Letters in Applied Microbiol. 4: 103-109.

Gorringe, A.R., Ashworth, L.A.E., Irons, L.I. and Robinson, A. 1985. Effect of monoclonal antibodies on the adherence of Bordetella pertussis to vero cells. FEMS Micro. Letters. 26: 5-9.

Gould, K. Ramirez-Ronda, C.H. Holmes, R.K. and Sanford, J.P. 1975. J. Clin. Infect. 56: 1364-1370.

Goulder, R. 1976. Attached and free-living bacteria in an estuary with abundant suspended solids. J. Applied Bacteriol. 43. 399-405.

Guentzel, M.N and Barry, L.J. 1975. Motility virulence factor for vibrio cholerae. Infect and Immune. 14: 890-897.

Handley, P.S., Carter, P.L and Fielding, J. 1984. Streptococcus salivarius carry either fibrils or fimbriae on the cell surface. J. Bacteriol. 157: 64-72.

Hanson, R.B. and Wiese, W.J. 1977. Heterotrophic activity associated with particulate size fractions in a salt-marsh estuary. Mar. Biol. 43: 321-330.

Harden, V.P. and Harris, J.D. 1953. The isoelectric point of bacteria cells. J. of Bacteriol. 65: 198-202.

Harris, R.H. and Mitchell, R. 1973. The role of polymer in microbial aggregation. Annu. Rev. Microbiol. 27: 27-50.

Harvey, R.W. and Young, L.Y. 1980. Enumeration of particle bound and unattached respiring bacteria in a salt marsh environment. Applied Environmental Microbiology. 40: 156-160.

Harvey, M., Forsberg, C.W., Beveridge, T., Pos, J. and Ogilvie, J.R. 1984. Methanogenic activity and structural characteristics of a micorbial biofilm on a needle-punctured polyester support. Applied and Environmental Microbiology. 48: 633-638.

Hattori, J and Furusaka, C. 1960. Chemical activities of E. coli absorbed on a resin. Biochem. J. 48: 831-837.

Hattori, J and Furusaka, C. 1961. Chemical activities of Azotobacter agile absorbed on resin. Biochem. J. 50: 312-315.

Hattori, R. and Hattori, T. 1963. Effect of the solid liquid interface on the life of microorganisms. Ecol. Rev. 16: 63-70.

Heeb, M.G., Costello, A.H. and Cabriel, O. 1982. Characterisation of a galactose specific lectin from Actinomyces viscosus by a model aggregation system. Infection and Immunity. 38: 993-1002.

Hobbie, J.E., Daley, R.J. and Jasper, S. 1977. Use of nuclepore filters for counting bacteria by fluorescence microscopy. Applied and Environmental Micorbiology. 33: 1225-1228.

Hsieh, Y.L and Merry, J. 1985. The adherence of Staphylococcus aureus. Staphylococcus epadermidis and E. coli on cotton polyester and their blends. J. of App. Bacteriol. 60: 535-544.

Hugh, R. and Leifson, E. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrate by various Gram-negative bacteria. Journal of Bacteriol. 66:24-26.

Humphrey, B., Kjelleberg, S. and Marshall, K.C. 1983. Responses of marine bacteria under starvation conditions at a solid-water interface. Allied and Environmental Microbiology. 45: 43-47.

Imam, S.H. and Gould, J.M. 1990. Adhesion of an amylolytic Arthrobacter species to starch containing plastic films. Applied and Environmental Microbiology. 56: 872-876.

Ishida, Y. and Kadota, H. 1981. Growth patterns and substrate requirements of naturally occuring obligate oligotrophs. Microbiol. Ecol. 7: 123-130.

Iriberri, J., Unanue, M., Ayo, B., Barcina, I. and Egea, E. 1990. Bacterial production and growth estimations from [^{3}H] thymidine incorporation for attached and free-living bacteria in aquatic systems. Applied and Environmental Microbiology. 56: 483-487.

Jack. T.R. and Zavic, J.E. 1977. The immobilisation of whole cells. Advances in Biochemical Engineering. 5: 125-145.

Jacques, M., Marrie, T.J. and Costerton, J.W. 1987. Microbiol colonisation of prosthetic devices. Microbiol. Ecol. 13: 173-191.

Jannasch, H.W. and Wirsen, C.O. 1977. Sci. Am. 236. 42.

Jannasch, H.W. and Pritchard, P.H. 1972. The role of inert particulate matter in the activity of aquatic microorganisms. Mem. First. Ital. Idrobiol. 289-308.

Jarmin, T.R. Deavian, L., Slocombe, S. and Righelato, R.C. 1978. Investigation of the effects of environmental conditions on the rate of exopolysaccharide synthesis in Azotobacter. J. of Gen. Microbiol. 107: 59-64.

Jeffrey, W.H. and Paul, J.H. 1986. Activity of an attached and free-living Vibrio species as measured by thymidina incorporation, p-indonitetrazolium reduction, and ATP/DNA ratio. Applied and Environmental Microbiology. 51: 150-156.

Jones, G.W., Abrahams, G.D. and Freter, R. 1976. Adhesive properties of Vibrio cholerae: Adhesion to isolated rabbit brush border membranes and haemagglutinating activity. Infection and Immunity. 14: 232-239.

Jones, G.W. and Freter, R. 1976. Adhesive properties Vibrio cholerae, nature of the interaction with rabbit brush border membranes and human erythrocytes. Infect and Immune. 14: 240-245.

Jones, M.J. and Epsteni, L. 1990. Adhesion of macroconidia to the plant surface and virulence of Nectria haematococca. Applied and Environmental Microbiology. 56: 3772-3778.

Jordan, T.L. and Stanley, J.T. 1976. Electron microscope study of succession in the periphyton communtiy of Lake Washington. Microbiol. Ecol. 2: 241-251.

Kang, K.S., Veeder, G.T., Mirrasoul, P.J., Kaneko, T. and Cottrell, I.W. 1982. Agar-like polysaccharide produced by Pseudomonas species. Production and basic properties. Applied and Environmental Microbiology. 43: 1086-1091.

Kefford, B., Kjelleberg, S. and Marshall, K.C. 1982. Bacterial scavenging: utilisation of fatty acids localised at a solid/liquid interface. Arch. Microbiol. 133: 257-260.

Kepkay, P.E., Schwinghamer, P., Willar, T. and Bower, A.J. 1986. Metabolism and metal binding by surface-colonising bacteria. Results of microgradient measurements. Applied and Environmental Microbiology. 51: 163-170.

King, E.O., Ward, M.E. and Raney, D.E. 1954. Two simple media for the demonstration of pyocyanin and flurescin. Journal of Laboratory and Clinical Medicine. 301-307.

Kirchman, D. and Mitchell, R. 1982. Contribution of particle bound bacteria to total microheterotrophic activity in five pounds and two marshes. Applied and Environmental Microbiology. 43: 200-209.

Kirchman, D. 1983. The production of bacteria attached to particles suspended in a freshwater pond. Limnol. Oceanogr. 28: 858-872.

Kolenbrander, P.E., Andersen, R.N. and Moore, L.V.H. 1990. Intrageneric coaggregration amongst strains of human oral bacteria: potential role in primary colonisation of the tooth surface. Applied and Environmental Microbiology. 56: 3890-3894.

Komiyama, K. and Gibbons, R.G. 1984. Interbacterial adhesion between Actinomyces viscosus and strains of Streptococcus. Infection and Immunity. 44: 86-90.

Kovacs, N. 1956. Identification Pseudomonas Pyocyanea by the oxidase reaction. Nature. 178: 703-708.

Kuznetsov, S.I., Dubinina, G.A. and Lapteva, N.A. 1979. Biology of oligotrophic bacteria. Ann. Rev.Microbiol. 33: 377-387.

Kjelleberg, S., Humphrey, B.A. and Marshall, K.C. 1983. Initial phases of starvation of bacteria at surfaces. App. and Env. Microbiol. 46: 978-984.

Lankford, C.E. 1960. Ann. N.Y. Acad. Sci. 88: 1203-1212.

Larsen, D.H., Dimmick, R.L. 1964. Attachment and growth of bacteria on surfaces of continuous culture vessels. J. of Bacteriol. 88: 1380-1387.

Lavie, S. and Stotzky, G. 1986. Adhesion of the clay minerals Montmolillanite, Kaolinite and Attapulgit reduces respiration of Histoplasma capsulatum. Applied and Environmental Microbiology. 51: 65-73.

Lawrence, J.R., Delaquis, P.J., Korber, D.R. and Caldwell, D.E. 1987a. Behaviour of Pseudomonas fluorescens within the hydrodynamic boundary layers of surface microenvironments. Microb. Ecol. 14: 1-14.

Lawrence, J.R. and Caldwell, D. 1987b. Behaviour of bacterial stream populations within the hydrodynamic boundary layers of surface microenvironments. Microb. Ecol. 14: 15-27.

LeChevallier, M.W., Hassenauerti, S.O., Camper, A.K. and Mcfetters, A. 1984. Disinfection of bacteria attached to granular activated carbon. Applied and Environmental Microbiology. 48: 918-923.

LeChevallier, M.W. and McFeters, G.A. 1985. Interactions between heterotrophic plate count bacteria and coliform organisms. Applied and Environmental Microbiololgy. 49: 1338-1341.

Liang, D.A., Sinclair, J.L., Mollory, L.M. and Alexander, M. 1982. Fate in model system of microbiol species of potential use in genetic engineering. Applied and Environmental Microbiololgy. 44: 704-714.

Lovell, C.R. and Konopka, A. 1985. Thymidine incorporation by free-living and particle bound bacteria in eutrophic dimictic lake. Applied Environmental Microbiology. 49: 501-504.

Mack, W.N., Mack, J.P. and Ackerson, A.D. 1975. Microbial film development in a trickling filter. Microbiol. Ecol. 2: 215-226.

MacFaddin, J.F. 1976. Biochemical tests for the identification of medical bacteria. Balitimore: Williams and Wilkins Company.

Magnusson, K.E., Stendahl, O., Tagesson, C., Edebo, L. and Johansson, G. 1977. The tendancy of smooth and rough Salmonella typhimurium bacteria and lipopolysaccharide to hydrophobic and ionic interaction, as studied in aqueous polymer two-systems. Acta. Pathologica Microbiologica-Scandinavia Sect. B 85: 221-228.

Mallory, L.M., Austin, B. and Colwell, R.R. 1977. Numerical taxonomy and ecology of oligotrophic bacteria isolated from the estuarine environment. Can. J. Microbiol. 23: 733-750.

Malmcrona-Friberg, K., Tunlid, A., Marden, P., Kjellberg, S. and Odham, G. 1986. Chemical changes in the cell envelope and poly-B-hydroxybutyrate during short term starvation of a bacterial isolate. Arch. Microbiol. 144: 340-345.

Marshall, K.C., Stout, R. and Mitchell, R. 1971a. Selective sorption of bacteria from seawater. Can. J. of Microbiol. 17: 1413-1416.

Marshall, K.C., Stout, R. and Mitchell, R. 1971b. Mechanisms of the initial events in the sorption of marine bacteria to surfaces. J. Gen. Microbiol. 68: 337-348.

Marshall, K.C. 1976. Interfaces in microbial ecology. Harvard University Press. Cambridge. Massachusetts.

Marshall, K.C. 1980. Bacterial adhesion in natural environments. In microbiol adhesion to surfaces ed. R.C.W. Berkely, J.M. Lynch, J. Melling, P.R. Rutter and B. Vincent. 187-196. England, Ellis Horwood.

Marshall, K.C. 1985. Mechanisms of bacterial adhesion at solid-water interfaces. In: Bacterial adhesion to surfaces, 133-161. ed. D.C. Savage and M. Fletcher. Plenum Publishing Corp. New York.

Marshall, P.A., Lobe, G.I., Cowan, M.M. and Fletcher, M. 1989. Response of microbial adhesives and biofilm matrix polymers to chemical treatments as determined by interference reflection microscopy and light section microscopy. Applied and Environmental Microbiology. 55: 2827-2831.

Marszalek, D.S., Gerchakov, S.M. and Vdeg, L.R. 1979. Influence of substrate composition on marine microfouling. Applied and Environmental Microbiology. 38: 987-995.

Massey, P. 1970. Antibiotic effects of sea water. Process Biochem. 5: 35-36.

McCourtie, J. and Douglas, L.J. 1981. Infection and Immunity. 32: 1234-1241.

McCourtie, J. and Douglas, L.J. 1984. Infection and Immunity. 45: 6-12.

McCourtie, J. and Douglas, L.J. 1985. Unequal distribution of adhesions within populations of Candida albicans. FEMS Microbiology Letters. 27: 111-115.

McCoy, W.F. and Costerton, J.W., 1982. Growth of sessile Sphaerotilus natan in a tubular recycle system. Applied and Environmental Microbiology. 43: 1491-1493.

Means, E.G. and Olson B.H. 1981. Coliform inhibition by bacteriocin like substances in drinking water distribution systems. Applied and Environmental Microbiology. 42: 506-512.

Meadows, P.S. 1971. The attachment of bacteria to solid surfaces. Archives of Microbiol. 75: 374-381.

Meinhard, S. 1985. Specific uptake rates of amino-acids by attached and free-living bacteria in mesotrophic lakes. Applied and Environmental Microbiology. 49: 1254-1259.

Meyer, J.L., Edwards, T. and Risley, R. 1987. Bacterial growth on dissolved organic carbon from a blackwater river. Microbial Ecology. 13: 13-29

Minato, H. and Suto, T. 1976. Technique for fractionation of bacteria in rumen microbial ecosystems. 1: Attachment of rumen bacteria to starch granules and elution of bacteria attached to them J. of Gen. Microbiol. 22: 259-276.

Mion, F., Jarmin, T.R. and Righelato, R.C. 1978. Biosynthesis of exopolysaccharide by Pseudomonas aeruginosa. J. of Bacteriol. 134: 418-427.

Mittelman, M.W. and Geesey, G.G. 1985. Copper-Binding characteristics of exopolymers from freshwater sediment bacteria. Applied and Environmental Micorbiology. 49: 846-851.

Moebus, K. 1972. Seasonal changes in antibacterial activity of North Sea water. Mar. Biol. (Berlin) 13: 1-13.

Moore, R.L. and Marshall, K.C. 1981. Attachment and rosette formation by hyphomicrobia. Applied and Environmental Microbiology. 42: 751-757.

Morse, S.A., Vaughan, P., Johnson, D. and Iglewski, B.H. 1976. Antimicrobial agents. Chemother. 10: 354-362.

Mouton, C., Reynolds, H. and Genco, R.J. 1978. Combined micromanipulation, culture and immunofluorescent techniques for isolation of coccal organisms comprising the "corn cob" configuration of human dental plaque. J. Bio. Buccale. 5: 321-322.

Neumann, A.W., Good, R.J., Hope, C.J. and Sejpal, M. 1974. An equation-of-state approach to determine surface tensions of low energy solids from contact angles. J. of Colloid and Interface Science. 49: 219-304

Neumann, A.W., Absolom, D.R., van Oss, C.J. and Zingg, W. 1979. Surface thermodynamics of leukocyte and platelet adhesion to polymer surfaces. Cell Biophysics. 1: 79-92.

Nikaido, H. and Nakae, T. 1980. The outer membrane of Gram-negative bacteria in microbial cell walls and membranes. 163-250. ed. H.J. Rogers, H.R. Perkins and J.B. Ward. London: Chapman and Hall.

Norde, W. and Lyklema, J. 1978. Adsorption of proteins from aqueous solutions on negatively charged polystyrene surfaces. In Ions in macromolecular and biological systems. ed. D.H. Everett. and B. Vincent. Proceedings of 29th symposium of Colston Research Society. 11-33.

Novitsky, J.A. and Morita, R.Y. 1976. Morphological characterisation of small cells resulting from nutrient starvation. Applied and Environmental Microbiology. 32: 617-622.

Novitsky, J.A. and Morita, R.Y. 1977. Survival of a psychrophilic marine vibrio under long term nutrient starvation. Applied and Environmental Microbiology. 33: 635-641.

Nuttall, D. and Parry, O.T. 1987. The effect of environmental factors on coliphages in the Welsh river Dee. Letters in Applied Microbiol. 4: 117-120.

Orstavik, D. 1977. Sorption of Steptococci to glass: effects of macromolecular solutes. Acta Pathologica et Microbiologica Scandinavica. 85: 47-53.

Oss, van C. J., Gillman, C.F. and Neumann, A.W. 1975. Phagocytic engulfment and cell adhesiveness as cellular surface phenomena. New York: Dekker.

Pace, G.W. and Righelato, P.C. 1980. Production of extracellular microbial polysaccharides. Advances in Biochem. Engineering. 15: 41-70.

Paul, J.H. 1984. Effects of antimetabolites on the adhesion of a estuarine Vibrio species to polystyrene. Applied and Environmental Microbiology. 48: 924-929.

Pederson, K. 1982. Method for studying microbial biofilms in flowing-water systems. Applied and Environmental Micorbiology. 43:6-13.

Pedros, A.C. and Brock, T.D. 1983. The importance of attachment to particles for planktonic bacteria. Arch. Hydrobiol. 98: 354-379.

Pethica, B.A. 1980. Microbial and cell adhesion. In Microbial adhesion to surfaces. ed. R.C.W. Berkeley, J.M. Lynch, J. Melling, P.R. Rutter and B. Vincent. 19-45. Chichester, England: Ellis Horwood.

Picologlou, B.F., Zelver, N. and Characklis, W.G. 1980. Biofilm growth and hydraulic performance. J. Hydraulic Div. Proc. Am. Soc. Civ. Eng. 106: 733-746.

Plummer, D.T. and James, A.M. 1961. Some physical investigations of the behaviour of bacterial surfaces III. The variation of the electrophoretic mobility and capsule size of Aerobacter aerogenes with age. Biochimica et Biophysica Acta. 53: 453-460.

Poindexter, J.S. 1981. The caulobacters, ubiquitous unusual bacteria. Microbiol. Reviews. 45: 123-179.

Pringle, J.H. and Fletcher, M. 1983. Influence of substratum wettability on attachment of freshwater bacteria to solid surfaces. Applied and Environmental Micorbiology. 45: 811-817.

Pringle, J.H., Fletcher, M. and Ellwood, D.C. 1983. Selection of attachment mutants during the continuous culture of Pseudomonas fluorescen and the relationship between attachment ability and surface composition. J. of Gen. Microbiol. 129: 2557-2569.

Pringle, J.H. and Fletcher, M. 1986. Influence of substratum hydration and absorbed macromolecules on bacteria attachment to surfaces. Applied and Environmental Microbiology. 1321-1325.

Quinn, J.P. 1984. The modification and evaluation of some cytochemical techniques for the enumeration of metabolically active heterotrophic bacteria in the aquatic environment. J. App. Bacteriol. 57: 51-58.

Ridgway, H.F., Wagner, R.M., Dawsey, W.T. and Lewin, R.A. 1975. Fine structure of cell envelope layers of Flexibacter polymorphus. Can. J. Microbiol. 21: 1733-1750.

Riley, T.V. and Hoffman, D. 1986. Interference with Haemophilus influenzea by other microorganisms. FEMS Microbiological Letters. 33: 55-58.

Robinson, R.W., Aldrich, H.C., Hurst, S.F. and Bleiwes, A.S. 1985. Role of the cell surface of Methano sarcina in cell aggregation. Applied and Environmental Microbiology. 49: 321-327.

Roger, V., Fonty, G., Komisarczuk-Bony, S. and Gouet, P. 1990. Effects of physicochemical factors on the adhesion to cellulose avicel of the ruminal bacteria Ruminococcus flavefaciens and Fibrobacter succinogenes. Applied and Environmental Microbiology. 56: 3081-3087.

Rollinger, Y. and Dott, W. 1987. Survival of selected bacterial species in sterilised activated carbon filters and biological activated carbon filters. Applied and Environmental Microbiology. 53: 777-781.

Rudd, T., Sterritt, R.M. and Lester, J.N. 1982. The use of extraction methods for the quantification of extracellular polymer production by Klebsiella aerogenes under varying cultural conditions. European J. of Applied Microbiol. and Biotech. 16: 23-27.

Rutter, P.R. and Abbott, A. 1978. A study of the interaction between oral Streptococci and hard surfaces. J. of Gen. Microbiol. 105: 219-266.

Samuelsson, M. and Kirchman, D.L. 1990. Degradation of adsorbed protein by attached bacteria in relationship to surface hydrophobicity. Applied and Environmental Microbiology. 56: 3643-3648.

Saz, A.K., Watson, S., Brown, S.R. and Lowery, D.L. 1963. Antimicrobial activity of marine waters. 1: Macromolecular nature of antistaphylococcal factor. Limmol. Oceanogr. 8: 63-67.

Scheraga, M., Meskill, M. and Litchfield, C.D. 1979. Analysis of methods for quantitative recovery of bacteria sorbed onto marine sediments 21-39. In C.D. Litchfield and A.L. Segfreid. ed. Methodology of biomass determinations and microbial activities. American Society for Testing and Materials. Washington, D.C.

Schlegel, H.G. 1986. General Microbiology. University Press. Cambridge.

Shands, J.W. 1966 Localisation of somatic antigen on Gram-negative bacteria using feritin antibody conjugants. Ann. of the N.Y. Acm of Science. 133: 292-298.

Shaw, D.J. 1970. Introduction to colloid and surface chemistry. 2nd edition. London: Butterworth.

Sieburth, J. 1975. Microbial seascapes. University Park Press. Baltimore

Sieburth, J. 1979. Sea microbes. Oxford University Press. New York.

Sjoblad, R.D. and Doetsch, R.N. 1982. Adsorption of polarly flaggellated bacteria to surfaces. Current Microbiology. 7: 191-194.

Sjollema, J., van der Mei, H.C., Uyen, H.N. and Busscher, H.J. 1990. Direct observations of cooperative effects in oral streptococcal adhesion to glass by analysis of the spatial arrangement of adhering bacteria. FEMS Microbiology Letters. 69: 263-270.

Skinner, Q.P., Adams, J.C., Beetle, A.A. and Roehrkasse, J.P. 1980. Change in bacterial populations down stream in Wyoming mountain drainage basin. Journal Range Management. 37: 269-274.

Slots, J. and Gibbons, R.J. 1978. Attachment of Bacterol. melaninogericus subsp asaccharolyticus to oral surfaces and the possible role in colonisation of the mouth. Infection and Immunity. 19: 254-264.

Stanley, J.T. 1968. Prosthecomicrobium and Ancalomicrobium: New Prothecate fresh-water bacteria. Journal of Bacteriol. 95: 1921-1942.

Stanley, P. 1968. Factors affecting the irreversible attachment of Pseudomonas aeruginosa to stainless steel. Can. J. Microbiol. 29: 1493-1499.

Stenstrom, T.A. 1989. Bacterial hydrophobicity, an overall parameter for the measurement of adhesion potential to solid surfaces. Applied and Environmental Microbiology. 55: 142-147.

Stevenson, I.L. 1962. Growth studies of Arthrobacter globiformus. Changes at macromolecular levels during growth. Can. J. Microbiol. 8: 655-661.

Stevenson, L.H. 1978. A case for bacterial dormancy in aquatic systems. Microbiol. Ecol. 4: 127-133.

Stewart, M., Wolfe, R.L. and Means, E.G. 1990. Assessment of bacteriological activity associated with granualar activated carbon treatment of drinking water. Applied and Environmental Microbiology. 56: 3822-3829.

Stotzky, G. 1972. Activity, ecology and population dynamics of microorganisms in soil. Crit. Rev. Microbiol. 2: 59:137.

Tamplin, M.L., Gauzens, A.L. Huq, A., Sack, D.A. and Colwell, R.R. 1990. Attachment of Vibrio cholerae serogroup 01 to zooplankton and phytoplankton of Bangladesh waters. Applied and Environmental Microbiology. 56: 1977-1980.

Tanford, C. 1979. Interfacial free-energy and the hydrophobic effect. Proceedings of the National Academy of Science. U.S.A. 76: 4174-4176.

Tewari, B.K. and Mishra, R.R. 1985. Population and productivity of heterotrophic bacteria in a fresh-water lake. ACTA Bot. INDICA. 13: 51-56.

Tomoichi, Y., Toshihiro, I., Tsuji, T., Kamata, Y., Ito, K. and Sasaki, S. 1978. Two trophic groups of bacteria, oligotrophs and eutrophs. Their distributions in fresh and seawater areas in central and Northern Japan. J. Gen. App. Microbiol. 24: 59-88.

Tranvik, K.J. and Hofle, M.G. 1987. Bacterial growth in mixed cultures in dissolved organic carbon from humic and clear waters. Applied and Environmental Microbiology. 53: 482-488.

Tranvik, L.J. 1990. Bacterioplankton growth on fractions of disolved organic carbon of different molecular weights from humic and clear waters. Applied and Environmental Microbiology. 56: 1672-1677.

Uhilinger, D.J. and White, D.C. 1983. Relationship between physiological status and formation of extra cellular polysacharide glycocalyx in Pseudomonas atlantica. Applied and Environmental Microbiology. 45: 64-70.

Vanhaecke, E., Remon, J., Moors, M., Raes, F., Rudder, D. and Peteghem, A. 1990. Kinetics of Pseudomonas aeruginosa adhesion to 304 and 306-L stainless steel: role of cell surface hydrophobicity. Applied and Environmental Microbiology. 56: 788-795.

Veldcamp, H. 1972. Mixed culture studies with the chemostat. J. App. Chem. Biotechnol. 22: 105-100.

Ward, M.E. and Watt, P.J. 1972. J Infect. Dis. 126: 601-605.

Ward, J.B. and Berkeley, R.C.W. 1980. The microbial surface and adhesion. In microbiol adhesion to surfaces. eds. R.C.W. Berkeley, J.M. Lynch., J. Melling, P.R. Ruter and B. Vincent. 47-66. Chichester. England. Ellis Horwood.

Wiebe, W.J. and Pomeray, L.R. 1972. Microorganisms and their association with aggregate and detritus in the sea: A microscopic study. Mem. First Ital idrobiol. Dott Marco de Marchi Pallanza Italy. 29: 325-352.

Wiese, W. and Rheninheimer, G. 1978. Scanning electron microscope and epifluorescence investigation of bacterial colonization of marine sediments. Microb. Ecol. 4: 175.

Wilkinson, J.F. 1958. The extracellular polysaccharides of bacteria. Bacterial Reviews. 22: 46-73.

Wilkinson, T.G. and Fowler, H.W. 1974. Wall growth in mixed bacterial cultures grown on methane. Biotechnol. Bioengin. 16: 251.

Williams, A.G. and Wimpenny, J.W.T. 1977. Exopolysaccharide production by Pseudomonas NCIB 11264 grown in batch culture. J. of Gen. Microbiol. 702: 13-21.

Williams, A.G. and Wimpenny, J.W.T. 1980. Extracellular polysaccharide biosynthesis by Pseudomonas NCIB 11264. Studies on precursor-forming enzymes and factors affecting exopolysaccharide production by washed suspensions. J. Gen. Microbiol. 116: 133-141.

Wrangstadh, M., Conway, P.L. and Kjelleberg, S. 1988. The role of an extracellular polysaccharide produced by the marine Pseudomonas species S9 in cellular detachment during starvation. Can. J. Microbiol. 35: 309-312.

Wright, S.F., Foster, J.G. and Bennett, O.L. 1986. Production and use of monoclonal antibodies for indentification of strains of Rhizobium trifolii. Applied and Environmental Microbiology. 52: 119-123.

Yanagita, T., Ichikawa, T., Tsuji, T., Kamata, Y., Ito, K. and Sasaki, M. 1978. Two trophic groups of bacteria , oligotrophs and eutrophs: Their distribution in fresh and sea water areas in the central nothern Japan. J. G. Microbiol. 24: 59-88.

Yoon, W.B. and Rosson, R.A. 1990. Improved method of enumeration of attached bacteria for study of fluctuation in the abundance of attached and free-living bacteria in response to diel variation in sea water turbidity. Applied and Environmental Microbiology. 56: 595-600.

Yoshimzu, M. and Kmura, I. 1983. Bacteriological study of a lake: 3 microflora of lake group Onuma. Bull Fac Fish Hokkaido Univ. 34: 361- 369.

Yoskikawa, H. and Takiguchi, T. 1979. Attached growth of Sphaerotilus natans in continuous flow apparatus and its grown inhibition by 9-D-arabinofuranosyladenine. Applied and Environmental Microbiology. 38: 200

Zambon, J.J., Huber, P.S., Meyer, A.E., Slots, J., Fornalik, M.S. and Baier, R.E. 1984. In situ identification of bacterial species in marine microfouling films by using an immunofluorescence technique. Applied and Environmental Microbiology. 48: 1214-1220.

Zvyagintsev, D.G., Gusev, V.S. and Guzeva, I.S. 1977. Relationship between adsorption of microorganisms and the stage of their development. Microbiology (USSR). 46: 245-249.

APPENDIX

TABLE 1.1 Identification Results

ORGANISM	GRAM STAIN	CELL MORPHOLOGY	MOTILITY LIQUID / SOLID MEDIUM / SURFACE		PIGMENT ON NA
Organism 1	-	cocco-bacillus	-	-	-
Organism 2	-	cocco-bacillus	-	-	-
Organism 3	-	bacillus	+	-	-
Organism 4	-	bacillus	+	-	-
Organism 5	-	bacillus	+	-	-
Organism 6	-	bacillus	+	-	P
Organism 7	-	bacillus	+	-	P
Organism 8	-	bacillus	+	-	-
Organism 9	-	bacillus	+	-	-
Organism 10	+	bacillus	+	-	Y
Organism 11	-	bacillus	-	-	Y / O
Organism 12	-	filamentous bacillus	+	+	Y / O
Organism 13	+	bacillus	+	-	-
Organism 14	+	cocci	+	-	-
Organism 15	-	bacillus	-	-	-
Organism 16	-	bacillus	+	-	-

KEY

P- PURPLE, Y- YELLOW, O- ORANGE

APPENDIX

TABLE 1.2

ORGANISM	INDOLE TEST	METHYL RED	V.P.	CITRATE UTILISATION
Organism 1	-	ND	ND	-
Organism 2	-	ND	ND	-
Organism 3	-	ND	-	+
Organism 4	-	ND	-	+
Organism 5	-	ND	-	+
Organism 6	ND	ND	ND	ND
Organism 7	ND	ND	ND	ND
Organism 8	+	-	+	+
Organism 9	+	-	+	+
Organism 10	-	ND	ND	ND
Organism 11	-	-	-	-
Organism 12	-	-	-	-
Organism 13	-	-	-	+
Organism 14	ND	ND	ND	ND
Organism 15	-	-	+	+
Organism 16	-	-	+	+

KEY

ND- NOT DETERMINED

APPENDIX

TABLE 1.3

ORGANISM	CATALASE TEST	UREASE TEST	H_2S + G.L.		OXIDASE TEST
Organism 1	+	+	-	-	-
Organism 2	+	+	-	-	-
Organism 3	+	+	-	+	+
Organism 4	+	+	-	+	+
Organism 5	+	+	-	+	+
Organism 6	ND	ND	ND	ND	ND
Organism 7	ND	ND	ND	ND	ND
Organism 8	+	-	+	+	+
Organism 9	+	-	+	+	+
Organism 10	+	-	ND	ND	-
Organism 11	+	-	-	+	+
Organism 12	-	-	+	+	-
Organism 13	+	-	-	-	-
Organism 14	+	ND	ND	+	ND
Organism 15	-	+	-	-	-
Organism 16	-	+	-	-	-

KEY

ND- NOT DETERMINED

APPENDIX

TABLE 1.4

ORGANISM	HUGH & LEIFSON'S TEST OX	F	KING'S A MEDIUM	KING'S B MEDIUM
Organism 1	+	-	ND	ND
Organism 2	+	-	ND	ND
Organism 3	+	-	+	+
Organism 4	+	-	+	+
Organism 5	+	-	-	+
Organism 6	+	-	ND	ND
Organism 7	+	-	ND	ND
Organism 8	+	-	ND	ND
Organism 9	+	-	ND	ND
Organism 10	ND	ND	ND	ND
Organism 11	+	-	ND	ND
Organism 12	+	-	ND	ND
Organism 13	+	-	ND	ND
Organism 14	-	+	ND	ND
Organism 15	+	-	ND	ND
Organism 16	+	+	ND	ND

KEY

ND- NOT DETERMINED

APPENDIX

TABLE 1.5

ORGANISM	POLYMER HYDROLYSIS			
	STARCH	CELLULOSE	CASEIN	DNA
Organism 1	-	-	-	-
Organism 2	-	-	-	+
Organism 3	-	-	-	-
Organism 4	+	-	-	-
Organism 5	+	-	+	-
Organism 6	+	-	-	-
Organism 7	+	-	-	-
Organism 8	+	-	-	+
Organism 9	+	-	-	+
Organism 10	+	-	ND	+
Organism 11	-	-	-	-
Organism 12	+	-	-	+
Organism 13	+	-	-	+
Organism 14	ND	ND	ND	ND
Organism 15	-	-	-	-
Organism 16	+	-	+	-

KEY

ND- NOT DETERMINED

APPENDIX

TABLE 1.6

ORGANISM	DIHYDROLASE & DECARBOXYLASE ACTIVITY		
	ARGININE DIHYDROLASRE	LYSINE DECARBOXYLASE	ORNITHINE DECARBOXYLASE
Organism 1	-	-	-
Organism 2	-	-	+
Organism 3	-	-	-
Organism 4	+	-	-
Organism 5	+	+	-
Organism 6	+	-	-
Organism 7	+	-	-
Organism 8	+	-	+
Organism 9	+	-	+
Organism 10	+	-	+
Organism 11	-	-	-
Organism 12	+	-	-
Organism 13	+	-	+
Organism 14	ND	ND	ND
Organism 15	-	-	-
Organism 16	+	-	-

KEY

ND- NOT DETERMINED

APPENDIX

TABLE 1.7

ORGANISM	FERMENTATION TESTS			
	GLUCOSE	MALTOSE	MANNITOL	ARABINOSE
Organism 1	ND	ND	ND	ND
Organism 2	ND	ND	ND	ND
Organism 3	ND	ND	ND	ND
Organism 4	ND	ND	ND	ND
Organism 5	ND	ND	ND	ND
Organism 6	+	-	-	-
Organism 7	+	-	-	-
Organism 8	+(G)	+	+	-
Organism 9	+(G)	+	+	-
Organism 10	ND	ND	ND	ND
Organism 11	+	+	+	-
Organism 12	ND	ND	ND	ND
Organism 13	ND	ND	ND	ND
Organism 14	ND	ND	ND	ND
Organism 15	+(G)	-	+	-
Organism 16	+(G)	-	+	+

KEY

+ - ACID

+(G) - ACID and GAS

ND - NOT DETERMINED

APPENDIX

TABLE 1.8

ORGANISM	FERMENTATION TESTS			
	XYLOSE	LACTOSE	INOSITOL	SUCROSE
Organism 1	ND	ND	ND	ND
Organism 2	ND	ND	ND	ND
Organism 3	ND	ND	ND	ND
Organism 4	ND	ND	ND	ND
Organism 5	ND	ND	ND	ND
Organism 6	-	-	-	+
Organism 7	-	-	-	+
Organism 8	-	-	-	+
Organism 9	-	-	-	+
Organism 10	ND	ND	ND	ND
Organism 11	-	-	-	-
Organism 12	ND	ND	ND	ND
Organism 13	ND	ND	ND	ND
Organism 14	ND	ND	ND	ND
Organism 15	+	+	+	+
Organism 16	+	+	-	+

KEY

ND - NOT DETERMINED

APPENDIX

TABLE 1.9

ORGANISM	FERMENTATION TESTS		
	MALONATE	ADONITOL	DULCITOL
Organism 1	ND	ND	ND
Organism 2	ND	ND	ND
Organism 3	ND	ND	ND
Organism 4	ND	ND	ND
Organism 5	ND	ND	ND
Organism 6	-	-	-
Organism 7	-	-	-
Organism 8	-	-	-
Organism 9	-	-	-
Organism 10	ND	ND	ND
Organism 11	-	-	-
Organism 12	ND	ND	ND
Organism 13	ND	ND	ND
Organism 14	ND	ND	ND
Organism 15	-	+	-
Organism 16	+	-	-

KEY

ND - NOT DETERMINED

APPENDIX

TABLE 1.10

ORGANISM	SPORE STAIN	MILK AGAR (10% NaCl)
Organism 1	ND	ND
Organism 2	ND	ND
Organism 3	ND	ND
Organism 4	ND	ND
Organism 5	ND	ND
Organism 6	ND	ND
Organism 7	ND	ND
Organism 8	ND	ND
Organism 9	ND	ND
Organism 10	ND	ND
Organism 11	ND	ND
Organism 12	ND	ND
Organism 13	+	ND
Organism 14	ND	+
Organism 15	ND	ND
Organism 16	ND	ND

KEY

ND - NOT DETERMINED

APPENDIX

TABLE 2.1 Percentage detachment of *Chromobacterium* in Tween-20 at 15°C for 30 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF TWEEN 20 (ppm) 200	100	50	25
50	57 (48)	52 (55)	34 (86)	31 (100)
100	62 (43)	60 (48)	39 (84)	35 (98)
150	71 (48)	62 (52)	45 (81)	36 (100)
200	70 (42)	61 (46)	46 (80)	39 (100)

TABLE 2.2 Percentage detachment of *Chromobacterium* in Tween-20 at 15°C for 60 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF TWEEN 20 (ppm) 200	100	50	25
50	87 (48)	72 (53)	64 (86)	41 (100)
100	90 (43)	84 (52)	70 (84)	49 (100)
150	93 (42)	85 (50)	80 (84)	61 (98)
200	93 (39)	88 (50)	75 (79)	59 (96)

Table 2.3 Percentage detachment of *Chromobacterium* in Tween 20 at 15°C for 90 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF TWEEN 20 (ppm) 200	100	50	25
50	96 (39)	87 (47)	71 (80)	39 (100)
100	95 (36)	91 (43)	76 (78)	58 (98)
150	94 (30)	92 (44)	79 (76)	60 (96)
200	96 (30)	92 (40)	80 (68)	67 (92)

APPENDIX

TABLE 2.4 Percentage detachment the coryneform in Tween-20 at 15°C for 30 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF TWEEN 20 (ppm) 200	100	50	25
50	76 (58)	75 (67)	53 (78)	45 (100)
100	70 (51)	69 (62)	50 (81)	48 (99)
150	79 (60)	68 (70)	59 (73)	50 (100)
200	68 (55)	60 (65)	52 (77)	46 (100)

TABLE 2.5 Percentage detachment of the coryneform in Tween-20 at 15°C for 60 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF TWEEN 20 (ppm) 200	100	50	25
50	80 (40)	78 (39)	52 (75)	51 (100)
100	87 (37)	79 (42)	50 (72)	46 (100)
150	86 (32)	73 (48)	57 (68)	52 (100)
200	90 (29)	82 (41)	60 (71)	56 (98)

Table 2.6 Percentage detachment of the coryneform in Tween-20 at 15°C for 90 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF TWEEN 20 (ppm) 200	100	50	25
50	94 (40)	95 (52)	79 (76)	62 (100)
100	98 (39)	97 (49)	88 (71)	68 (99)
150	97 (35)	92 (57)	80 (69)	70 (100)
200	96 (33)	90 (46)	85 (74)	71 (98)

APPENDIX

TABLE 2.7 Percentage detachment of Chromobacterium in EGTA at 15°C for 30 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF EGTA (mg) 20	10	5	1
50	91 (31)	90 (46)	56 (59)	48 (88)
100	95 (29)	92 (37)	60 (62)	53 (81)
150	95 (36)	96 (42)	65 (64)	60 (90)
200	98 (31)	93 (40)	68 (67)	59 (85)

TABLE 2.8 Percentage detachment of Chromobacterium in EGTA at 15°C for 60 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF EGTA (mg) 20	10	5	1
50	95 (34)	92 (41)	84 (61)	68 (79)
100	97 (30)	94 (48)	80 (57)	64 (73)
150	98 (28)	95 (39)	75 (53)	58 (77)
200	98 (25)	91 (33)	72 (59)	68 (71)

Table 2.9 Percentage detachment of Chromobacterium in EGTA at 15°C for 90 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF EGTA (mg) 20	10	5	1
50	98 (29)	95 (36)	89 (47)	63 (61)
100	99 (21)	93 (34)	83 (45)	69 (67)
150	98 (20)	97 (36)	81 (49)	74 (56)
200	96 (25)	95 (31)	88 (42)	71 (55)

APPENDIX

TABLE 2.10 Percentage detachment the coryneform in EGTA at 15°C for 30 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF EGTA (mg) 20		10		5		1	
50	90	(31)	72	(45)	68	(51)	57	(80)
100	92	(37)	74	(47)	64	(58)	53	(74)
150	96	(35)	77	(41)	70	(55)	62	(78)
200	93	(29)	80	(39)	63	(53)	59	(72)

TABLE 2.11 Percentage detachment of the coryneform in EGTA at 15°C for 60 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF EGTA (mg) 20		10		5		1	
50	97	(38)	86	(40)	73	(50)	69	(62)
100	93	(29)	88	(39)	80	(45)	63	(71)
150	97	(31)	91	(31)	89	(48)	70	(63)
200	98	(25)	93	(35)	85	(44)	73	(68)

Table 2.12 Percentage detachment of the coryneform in EGTA at 15°C for 90 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF EGTA (mg) 20		10		5		1	
50	98	(21)	96	(30)	87	(40)	79	(65)
100	98	(25)	98	(29)	82	(37)	82	(62)
150	97	(27)	95	(35)	89	(41)	80	(56)
200	99	(23)	91	(29)	88	(37)	76	(59)

APPENDIX

TABLE 2.13 Percentage detachment of Chromobacterium in Tween-20 at 37°C for 30 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF TWEEN 20 (ppm) 200	100	50	25
50	79 (63)	72 (80)	72 (100)	57 (100)
100	88 (62)	81 (85)	78 (100)	54 (98)
150	92 (59)	89 (81)	81 (100)	63 (100)
200	94 (57)	83 (72)	81 (96)	61 (98)

TABLE 2.14 Percentage detachment of Chromobacterium in Tween-20 at 37°C for 60 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF TWEEN 20 (ppm) 200	100	50	25
50	95 (59)	92 (75)	80 (100)	61 (100)
100	97 (62)	90 (72)	81 (100)	69 (100)
150	100 (58)	95 (70)	89 (100)	73 (100)
200	98 (59)	91 (73)	84 (99)	75 (100)

Table 2.15 Percentage detachment of Chromobacterium in Tween 20 at 37°C for 90 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF TWEEN 20 (ppm) 200	100	50	25
50	96 (51)	89 (63)	73 (100)	63 (100)
100	98 (55)	81 (69)	76 (100)	68 (100)
150	98 (50)	92 (72)	88 (98)	75 (100)
200	91 (59)	89 (78)	86 (98)	69 (98)

APPENDIX

TABLE 2.16 Percentage detachment the coryneform in Tween-20 at 37°C for 30 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF TWEEN 20 (ppm) 200	100	50	25
50	79 (67)	73 (86)	53 (100)	50 (100)
100	83 (65)	60 (83)	59 (100)	55 (100)
150	85 (62)	74 (79)	68 (100)	36 (100)
200	82 (55)	70 (81)	66 (100)	39 (100)

TABLE 2.17 Percentage detachment of the coryneform in Tween-20 at 37°C for 60 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF TWEEN 20 (ppm) 200	100	50	25
50	82 (66)	75 (80)	59 (100)	57 (100)
100	89 (61)	80 (72)	64 (100)	52 (100)
150	91 (58)	83 (75)	72 (100)	65 (100)
200	93 (49)	85 (73)	65 (98)	66 (100)

Table 2.18 Percentage detachment of the coryneform in Tween-20 at 37°C for 90 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF TWEEN 20 (ppm) 200	100	50	25
50	83 (56)	71 (76)	71 (100)	49 (100)
100	89 (51)	73 (70)	62 (100)	45 (100)
150	94 (48)	90 (65)	70 (98)	61 (100)
200	91 (43)	87 (62)	67 (98)	54 (100)

APPENDIX 2

TABLE 2.19 Percentage detachment of Chromobacterium in EGTA at 37°C for 30 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF EGTA (mg) 20	10	5	1
50	89 (51)	80 (62)	55 (76)	52 (98)
100	91 (47)	85 (57)	58 (81)	57 (98)
150	94 (43)	91 (58)	63 (77)	62 (92)
200	92 (40)	85 (54)	61 (72)	59 (90)

TABLE 2.20 Percentage detachment of Chromobacterium in EGTA at 37°C for 60 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF EGTA (mg) 20	10	5	1
50	94 (42)	92 (52)	87 (64)	63 (92)
100	98 (42)	95 (60)	82 (78)	60 (90)
150	98 (37)	96 (48)	86 (63)	67 (86)
200	98 (33)	84 (42)	79 (60)	72 (90)

Table 2.21 Percentage detachment of Chromobacterium in EGTA at 37°C for 90 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF EGTA (mg) 20	10	5	1
50	96 (41)	90 (51)	76 (68)	69 (92)
100	98 (35)	96 (49)	80 (64)	67 (87)
150	98 (39)	95 (41)	83 (55)	78 (82)
200	98 (37)	94 (46)	86 (59)	73 (80)

APPENDIX

TABLE 2.22 Percentage detachment the coryneform in EGTA at 37°C for 30 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF EGTA (mg) 20	10	5	1
50	92 (52)	81 (58)	76 (72)	67 (89)
100	92 (49)	76 (58)	71 (66)	62 (92)
150	98 (45)	80 (53)	70 (65)	59 (81)
200	94 (36)	78 (49)	73 (59)	54 (85)

TABLE 2.23 Percentage detachment of the coryneform in EGTA at 37°C for 60 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF EGTA (mg) 20	10	5	1
50	96 (40)	82 (51)	76 (63)	74 (87)
100	98 (37)	89 (49)	84 (55)	76 (85)
150	98 (31)	95 (41)	91 (52)	83 (79)
200	98 (31)	94 (44)	89 (56)	82 (81)

Table 2.24 Percentage detachment of the coryneform in EGTA at 37°C for 90 mins, and percentage of cell viability on detachment.

INCUBATOR (rpm)	CONCENTRATION OF EGTA (mg) 20	10	5	1
50	98 (38)	94 (46)	83 (58)	79 (79)
100	98 (32)	97 (41)	82 (61)	76 (77)
150	99 (32)	98 (36)	87 (75)	80 (84)
200	97 (30)	95 (40)	80 (68)	83 (80)

APPENDIX

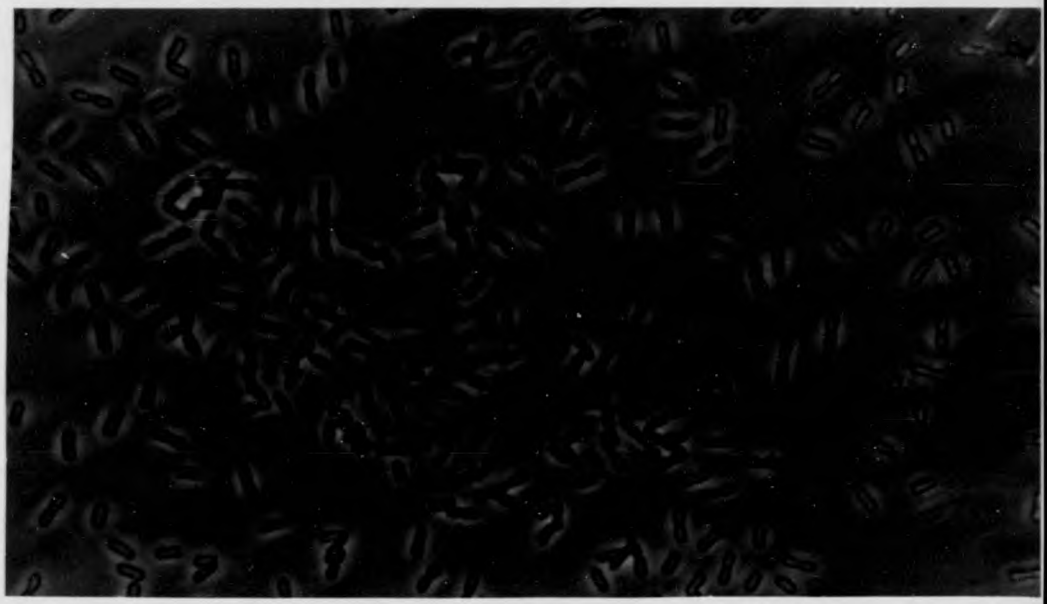
TABLE 3.1 Effect of Silicoset on bacterial cell viabilty, growth and attachment ability. The bacteria were grown and attached in batch culture in a glucose minimal medium.

BACTERIA	ATTACHMENT RESULTS	
	NO SILICOSET	SILICOSET
Aeromonas	0.3461 (0.0404)	0.3439 (0.0419)
Chromobacterium	0.2387 (0.0296)	0.2410 (0.0314)
Staphylococcus	0.4920 (0.0562)	0.5126 (0.0591)
coryneform	0.3698 (0.0456)	0.3591 (0.0433)
Acinetobacter	0.0000	0.0000
Pseudomonas	0.2983 (0.0347)	0.3025 (0.0368)

N.B.

The growth of the bacteria in the media with or without Slicoset was not influenced by the presence of the Silicoset. The viability of the bacterial cells grown in these media also did not vary during these experiments.

(a)



(b)

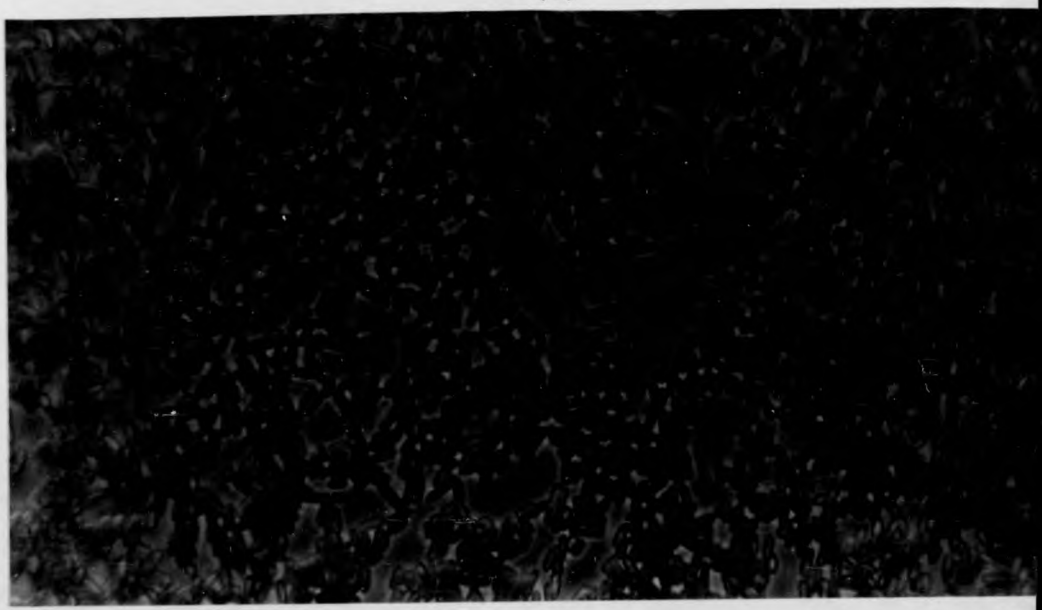


FIGURE 1

(a) The biofilm obtained after the growth and attachment of *Aeromonas* in glucose Medium 4 in batch culture at 15°C.

(b) The biofilm obtained after the growth of *Aeromonas* in continuous culture in mannose Medium 6 at 15°C and the subsequent attachment of *Aeromonas* in batch at 25°C.

(a)

(b)

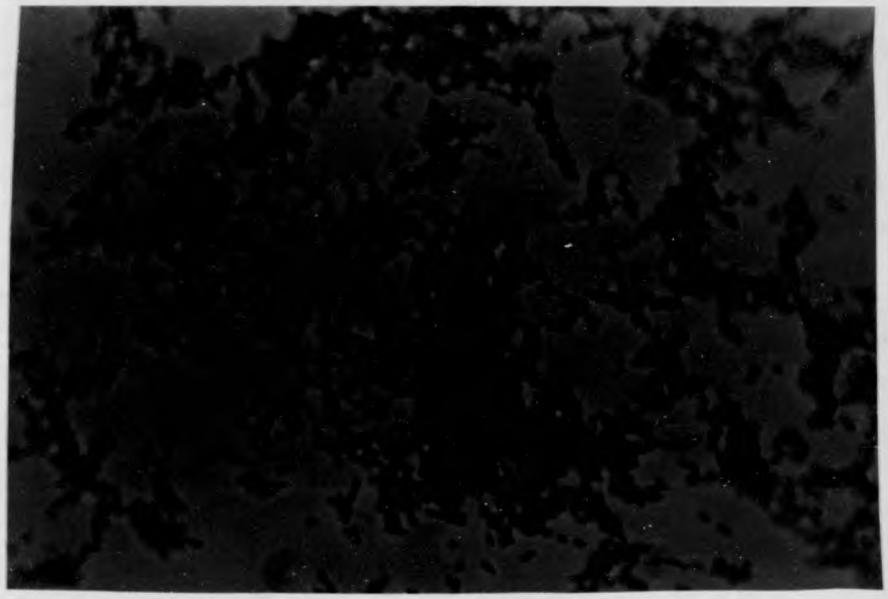
FIGURE 2

(a) The biofilm obtained after the attachment of a mixed suspension of Chromobacterium with the coryneform. The bacteria were grown in batch culture in mannose Medium 5 at 15°C and attached in the same medium at 37°C

(b) The biofilm obtained after the growth and mixed suspension attachment of the coryneform with Staphylococcus. Both bacteria were grown in batch in glucose Medium 8 at 15°C. The attachment also occurred in batch at 15°C.

APPENDIX

(a)



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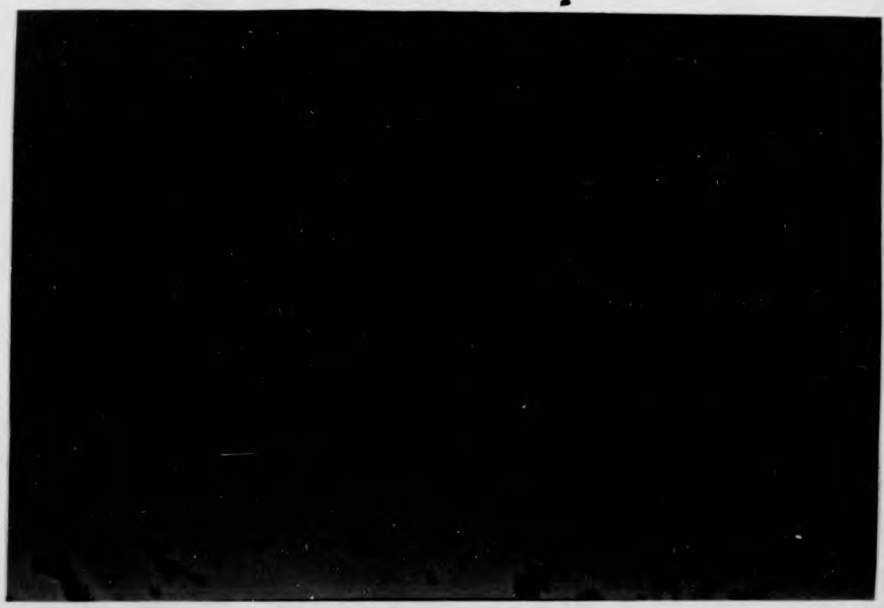

FIGURE 3

(a) The biofilm obtained after the attachment of Aeromonas, Chromobacterium and the coryneform in the model system.

(b) The biofilm obtained after the attachment of Aeromonas, Chromobacterium, the coryneform and Acinetobacter in the model system.

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DEGREE .....

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